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TUMOUR NECROSIS FACTOR-ALPHA (TNFα) IN AVIAN EMBRYOGENESIS

by

MICHAEL ANTHONY WRIDE 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Physiology

Edmonton, Alberta

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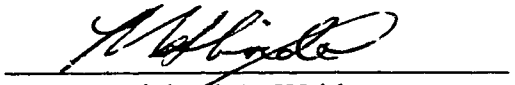

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To Whom It May Concern,

I co-authored with Mike A. Wride, a paper entitled "Distribution of TNFα-Like Proteins Correlates with Some Regions of Programmed Cell Death in the Chick Embryo" which was published in the International Journal of Developmental Biology, 38: 673-682, 1994. I give Mike A. Wride unconditional permission to use this paper in his Doctor of Philosophy thesis.

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Sanders E J, Prasad S, Hu N, Wride M A. Cell death in the gastrulating chick embryo: potential roles for tumour necrosis factor-alpha (TNF-alpha). (submitted to Developmental Dynamics).

Sanders E J, Wride MA. (1996). Roles for growth and differentiation factors in avian development. *Poultry Sci.*, (in press).

Sanders E J, Wride M A. (1995). Programmed cell death in development. *Int. Rev. Cytol.*, 163: 105-173.

Wride M A, Sanders E J. (1995). Potential roles for tumour necrosis factorα during embryonic development. *Anat. Embryol.* 191: 1-10.

Wride M A, Lapchak P H, Sanders E J. (1994). Distribution of TNFα-like proteins correlates with some regions of programmed cell death in the chick embryo. *Int. J. Dev. Biol.*, 38: 673-682.

Wride M A, Sanders EJ. (1993). Expression of tumor necrosis factor-alpha (TNFα) cross-reactive proteins during early chick embryo development. *Dev. Dynamics*, 198: 225-239.

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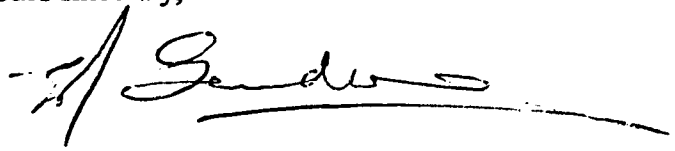

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Yours sincerely,

Ninghe Hu

"I will confess that I frequently have the feeling in my experimental work of holding a dialogue with someone who is considerably brighter than I am"

Hans Spemann, 1928[1]

"The scientist has a lot of experience with ignorance and doubt and uncertainty... When a scientist doesn't know the answer to a problem, he is ignorant. When he has a hunch as to what the result is, he is uncertain. And when he is pretty darn sure of what the result is going to be, he is still in some doubt. Scientific knowledge is a body of statements of varying degrees of uncertainty - some most unsure, some nearly sure, but none absolutely certain"

Richard P. Feynman, 1955[2]

---

[1] *Zur Theorie der tierischen Entwicklung, Rektoratsträde. Freiburg: Speyer u Kaerner.*

[2] *The Value of Science. A public address given to the Autumn meeting of the National Academy of Sciences, U.S.A. In: "What Do You Care What Other People Think?". Further Adventures of a Curious Character. HarperColiinsPublishers.*

For my Mum Mary, my Dad Eric, and my sister Sally.

Although we have been miles apart, you have always been in my thoughts. I thank you for your support and encouragement through all the years.

And to my fiancée Catherine

with all my love.

ABSTRACT

Tumour necrosis factor α (TNFα) is a member of the family of molecules called cytokines, which have important roles to play in many aspects of physiology and pathophysiology. Considering the relevance of these roles to many processes occurring during embryonic development, the possibility that TNFα is expressed in the developing chick embryo was investigated. TNFα immunoreactivity was present in several tissues undergoing programmed cell death during development as well as in tissues undergoing major cellular re-organisation. In addition to a TNFα immunoreactive protein of approximately 17 kDa in size, a number of higher molecular weight TNFα cross-reactive proteins were identified. TUNEL, a technique that allows the detection of DNA fragmentation in dying cells, showed that in several developing tissues including parts of the nervous system, the lens, and the limbs, the presence of TNFα immunoreactivity coincided with cell death. In the developing eye lens, patterns of TNFα and TNF receptor immunoreactivity correlated with a number of morphogenetic events occurring during lens fibre cell differentiation, including lens fibre cell denucleation. Using chick lens epithelial cell cultures, it was shown that the number of degenerating nuclei per unit area of differentiating lens epithelial cells or 'lentoids" (equivalent to lens fibre cells) was influenced significantly by the addition of various concentrations of TNFα, anti-TNFα, anti-TNFR1 and anti-TNFR2 to the cell culture medium. These studies demonstrate that TNFα-related proteins are expressed during early avian development in a number of tissues that are undergoing important morphogenetic processes. Furthermore, TNFα appears to be one of the factors involved in the degeneration of lens fibre cell nuclei during differentiation of the chick lens.

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A resident of Pembina Hall, who deserves special thanks, is my fiancée Catherine Fear, whom I met after only a few weeks of my arrival in Canada, and to whom this thesis is jointly dedicated. I would like to thank her for her understanding, as a fellow graduate student, also due to finish in Spring 1996, her encouragement and faith in me, for being my Excel spreadsheet advisor on numerous occasions, and for being my very best friend. This thesis would have been much more difficult, if not impossible, to complete without her constant cheerfulness, encouragement, and inspiration. The Fear and Boyd families of Toronto also deserve special mention for providing me with a home away from home and for taking me in during Christmas 1991, 1992, and 1994.

This thesis is also jointly dedicated to my family in Somerset, England. Through difficult times over the last ten years because of my father's ill health, due to kidney failure, they have been constantly supportive. My Dad had a kidney transplant in August 1995, and with a little luck will be able to attend my convocation and my wedding in June, 1996. Here's to the future.....

Lastly, but by no means least, I would like to acknowledge the invaluable financial support of the Alberta Heritage Foundation for Medical Research for my studentship and the annual research allowance that allowed me to travel to meetings; the University of Alberta for my Ph.D. scholarship, The Andrew Stewart Memorial Prize, and The Marie Louise Imrie Travel Award; and the Medical Research Council of Canada for an operating grant to Dr Esmond J. Sanders, in whose laboratory this work was carried out.

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LIST OF ABBREVIATIONS

AA, arachidonic acid

AEC, 3-amino-9-ethyl carbazole

ANOVA, analysis of variance

BCIP, 5-bromo-4-chloro-3-indoyl phosphate

BSA, bovine serum albumin

CAM, cell adhesion molecule

cAMP, cyclic adenosine monophospate

CAPK, ceramide activated PK

CAT, chloramphenicol acetyltransferase

cDNA, complementary DNA

CMF, calcium magnesium free

CNS, central nervous system

CNTF, ciliary neurotrophic factor

$cPLA_2$, cytoplasmic Ca^{2+}-dependent PLA_2

CREB, cAMP response element binding

CRP, cross-reactive protein

CSF, colony stimulating factor

CTL, cytotoxic T-lymphocyte

DAG, diacylglycerol

DDW, distilled de-ionised water

DMF, di-methyl formamide

DNA, deoxyribonucleic acid

DNase, deoxyribonucleic acidase

DTT, dithiothreitol

dUTP, deoxyuridine triphosphate

ECL, enzyme chemiluminescence

ECM, extracellular matrix

ED, embryonic day

EDTA, ethylenediamine-tetraacetic acid

EGF, epidermal growth factor

EGF-R, epidermal growth factor receptor

EGTA, ethyleneglycol-bis (b-amino ethyl ether) N,N'-tetra-acetic acid

Epo, erythropoeitin

FCS, foetal calf serum

FGF, fibroblast growth factor

FITC, fluorescein isothiocyanate

G-CSF, granulocyte colony-stimulating factor

GAG, glycosaminoglycan

GM-CSF, granulocyte-macrophage colony-stimulating factor

HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

5-HPETE, 5-hydroperoxyeicosatetraenoic acid

HSP, heat shock protein

ICAM, intercellular adhesion molecule

ICE, interleukin-1β-converting enzyme

IFAP, intermediate filament associated protein

IFN, interferon

Ig, immunoglobulin

IGF, insulin-like growth factor

IL, interleukin

IRF, interferon-regulatory-factor

LIF, leukaemia inhibitory factor

LKT, leukotriene

LPS, lipopolysaccharide

LT, lymphotoxin

MAPK, mitogen activated protein kinase

MHC, major histocompatibility complex

MIF, macrophage-migration inhibitory factor

MIP, major intrinsic protein

MnSOD, manganous superoxide dismutase

mRNA, messenger RNA

MS, multiple sclerosis

my, myelencephalic blebs mice

NADPH, nicotinamide adenine dinucleotide phosphate

NBT, nitroblue tetrazolium

Neut SMase, neutral SMase

NGF, nerve growth factor

NK, natural killer

NKC, natural killer cell

NO, nitric oxide

$p75^{NGFR}$, p75 low affinity nerve growth factor receptor

PAGE, polyacrylamide-gel-electrophoresis

Pax, paired box gene

PBS, phosphate buffered saline

PC-PLC, phosphatidylcholine-specific phospholipase C

PCR, polymerase chain reaction

PDGF, platelet derived growth factor

PG, prostaglandin

PK, protein kinase

PK-C, protein kinase C

PL-D, phospholipase D

PLA_2, phospholipase A_2

PMA , phorbol 12-myristate

PMSF, Phenylmethylsulphonyl fluoride

PTDA, phosphatidic acid

RA, retinoic acid

RNA, ribonucleic acid

RS, repressor site

RT-PCR, reverse transcriptase-polymerase chain reaction

SCF, stem cell factor

SDS, sodium-dodecyl-sulphate

Sey, small eye gene mutation

sHSP, small heat shock protein

SIRS, systemic inflammatory response syndrome

sm, skimmed milk

SM, sphingomyelin

SMase, sphingomyelinase

SSC, saline sodium citrate

sTNFR, soluble TNFR

TBS, tris-buffered saline

TdT, terminal deoxynucleotide transferase

TGF, transforming growth factor

TNFα, tumour necrosis factor alpha

TNFα-CRPs, tumor necrosis factor alpha-cross-reactive proteins

TNFR, TNF receptor

TRAF, TNFR associated factor

TRAK, TNFR associated kinase

TRAP, TNFR associated protein

TTBS, TBS with tween

TUNEL, terminal deoxynucleotide transferase mediated dUTP-biotin nick-end labelling

Tween, polyaxyethylenesorbitan monolaurate

VCAM, vascular cell adhesion molecule

VCAM-1, vascular CAM-1

CHAPTER 1

INTRODUCTION[1]

1.1. Rationale

During the early stages of embryonic development, all organisms utilise very similar morphogenetic mechanisms and early embryos from species as diverse as frogs and humans look remarkably similar to each other. The chick embryo was first used as a model for studying the events of development by Aristotle in the fourth century B.C. and the early chick embryo continues to provide an excellent model system in which to study many phenomena associated with cellular differentiation and interactions. Because of these advantages, it is the chick embryo which is used as the model system in this thesis.

Fertilisation, the fusion of egg and sperm, is the stimulus for the cascade of activity that results in the formation of the millions of cells of which the embryo is composed. A subsequent event, the process of gastrulation, is possibly the most important of all. Professor Lewis Wolpert[2], has argued that: "It is not birth, marriage, or death, but gastrulation, which is truly the most important time in your life!" During gastrulation, the two layered embryo is transformed into a three layered structure by formation of the mesoderm, which is the middle layer of the embryo. Subsequently, the final shaping of the embryo and the formation of organs begins to take place through complex processes of cell differentiation, migration, adhesion, and even death.

The death of cells during normal development is a paradox. Why should a constructive process like development include an apparently destructive and wasteful process like cell death? Cell death is important, for example, in the development of the limbs in which the hands and feet emerge as flat paddles and the digits are formed from the death of the cells between them (a reduction in this cell death in ducks results in their webbed feet). It is also important during development of the nervous system in which the matching of the number of

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[1] *A version of part of this Chapter was first presented during a speech by the author on his acceptance of The Andrew Stewart Memorial Prize awarded by The University of Alberta in May 1994.*

[2] *In: The Triumph of the Embryo. Wolpert L. (1991). Oxford University Press.*

motoneurons with the number of muscle fibres is controlled by the selective death of a large percentage of neurons; a kind of "survival of the fittest" at a cellular level.

One means by which cells communicate with each other during development, and throughout the rest of life, is by the production of hormones and growth factors. These factors may be soluble or membrane bound and when expressed in certain tissues at certain times they can cause cells to change their behaviour in certain ways. It is these proteins which are, in effect, the words and sentences in the language of the cells during the development of the embryo. The group of factors known as cytokines have received only superficial attention to date regarding their possible functions in development, in which they may have important roles to play in the modulation of cell behaviour. Cytokines are involved in the regulation of cell interactions within the immune system; their production is vital in the response of the body to microbial invasion, injury and cancer; and they have a major role to play in inflammatory conditions, such as arthritis. Tumour necrosis factor α (TNFα) is one of these factors and it contributes towards the development of the symptoms of fever and has a role in the death of cells in the immune system, during the immune response. It is not clear which proteins tell cells to die during development and in this thesis the potential role of TNFα during development, on events including cell death and related processes, is investigated.

Studies that investigate the role of proteins capable of causing cell death during development could open the door, not only to a better understanding of the fundamental process of development, but may also provide a better understanding of the mechanisms behind diseases such as cancer. For example, it is possible that some tumours may form in particular tissues not only due to an excess of cell proliferation, but also due to a reduction in naturally occurring cell death in those tissues. The potential ability to reactivate embryonic cell death mechanisms in tumours may be a useful and important method for bringing about tumour regression. In addition, certain neurodegenerative diseases may be due to the reactivation of embryonic cell death mechanisms in adult nervous tissue. An understanding of these embryonic mechanisms and the possibility of gaining the ability to modulate them may be useful in the treatment of these neurodegenerative diseases.

1.2. Objectives

The objectives of this thesis are as follows:

1. To develop immunostaining protocols that allow investigations into the distribution of TNFα immunoreactivity during chick embryo development and to understand more about the size and potential nature of the immunoreactive proteins identified.

2. To develop a hypothesis proposing specific roles for TNFα during embryonic development, based on its known roles in physiology and pathophysiology; e.g. immunity and inflammation, and its spatio-temporal patterns of expression during embryonic development.

3. To test the hypothesis developed using a well understood developing system, which possesses TNFα immunoreactivity and in which TNF receptors are expressed.

4. To discuss the implications of the results and to suggest avenues for future research.

1.3. Organisation

In order to obtain these objectives, the thesis is organised as follows. Part I is comprised of Chapters 2 and 3. Chapter 2 provides a comprehensive review of the literature pertaining to TNFα and its receptors and introduces the emerging family of TNF ligand- and receptor-related molecules. This Chapter also outlines the mechanisms of TNFα-mediated signalling, reviews the literature pertaining to the role of TNFα in disease, in the physiology and pathophysiology of the nervous system, in the immune system, and in reproduction,

including evidence for TNFα and TNFR expression in embryos. Since, the chick embryo is the model system used for the studies described in this thesis, the literature pertaining to avian TNFα-like factors is also reviewed. In Chapter 3, the literature is reviewed pertaining to the effects of TNFα in the immune system, and during inflammation, on such processes as programmed cell death (apoptosis), cellular proliferation and differentiation, extracellular matrix (ECM) remodelling, and on cell adhesion molecule (CAM) and integrin expression. All of these effects of TNFα are considered to be relevant to its potential involvement in embryonic development and it is proposed that TNFα may have three distinct roles during embryonic development, which are analogous to its roles in the immune system and during inflammation. These roles include effects on programmed cell death; on growth and differentiation; and on the remodelling of the ECM and the regulation of CAMs and integrins. This hypothesis is used as the basis for the approach used in subsequent Chapters of the thesis. Special attention is given throughout to the potential effects of TNFα on programmed cell death, and related processes, during embryonic development.

Part II is comprised of Chapters 4 and 5, which are concerned with immunolocalisation studies. In Chapter 4, immunohistochemistry and Western blotting techniques are used to gain an understanding of the spatio-temporal expression patterns of TNFα-related proteins, during early avian development. This information is then used to speculate further upon the potential roles for TNFα in development. It is apparent from this initial study that in several tissues in which TNFα is expressed, programmed cell death, or related processes, are also occurring. Of particular note, in this regard, is the intense and highly reproducible observation of TNFα immunoreactivity in the developing lens. In Chapter 5, the observation that TNFα is expressed in some tissues undergoing programmed cell death is investigated further. A technique is used that allows identification of cells undergoing cell death in tissue sections and, in several regions, this cell death correlates with the presence of TNFα immunoreactivity. In addition, a bioassay for TNFα is used, which reveals that chick embryo homogenate contains TNFα-like bioactivity.

Part III is comprised of Chapters 6 and 7. Chapter 6 provides an introduction to the development of the eye lens, which is used as a model system in Chapter 7. The main emphasis

in Chapter 6 is on recent advances in the understanding of molecular and cellular aspects of lens development. Chapter 7 focuses in on functional studies carried out on the developing lens, which is introduced in Chapter 6. The expression of a TNFα-related protein and TNF receptors is described and a cell culture system is introduced that allows functional studies to be carried out, which provide direct evidence of a role for TNFα in the degeneration of lens fibre cell nuclei during lens fibre cell terminal differentiation.

Finally, Chapter 8, provides a general discussion and conclusions and suggests a number of avenues for future research.

PART I: LITERATURE REVIEW AND HYPOTHESIS

CHAPTER 2

LITERATURE REVIEW[1]

2.1. Introduction to TNFα

2.1.1. Historical background

It has been recognised, since the 1700's, that certain cancer patients, who develop concurrent bacterial infections, experience remissions of their malignant disease. However, it took until the end of the 19th century before William B. Coley, a New York surgeon, was able to make use of this observation in a clinical sense (Wiemann and Starnes, 1994). He looked back through the medical literature, following the death of his first cancer patient, a young girl who had a sarcoma of the right arm, and located a seven year old record of a patient who had experienced remission, and complete recovery, from an inoperable sarcoma of the neck, under the influence of an erysipelas infection (a superficial streptococcal infection of the skin). A large number of other publications described similar cases. This astounded Coley, who set to work at attempts to deliberately bring about erysipelas, in his cancer patients, in order to bring their malignancies under control. His first attempt, though fraught with side effects, resulted in the recovery of the patient, until a relapse, as a result of the malignant nature of the tumour, resulted in the death of the patient 8 years later. Through much trial and error, Coley managed to refine his bacterial toxins until he obtained reproducible therapeutic effects in the clinic. An important addition to the panel of toxins used was that of *Bacillus prodigiosus* (now known as *Serratia marcescens*), which contained the important ingredient endotoxin. The mixture of toxins became known as "Coley's toxins" and Coley, and his contemporaries, obtained cure rates of better than 10% (Wiemann and Starnes, 1994).

However, it was still not clear as to why Coley's toxins were successful. Did they act directly or did they stimulate the production of some factor(s) effective in bringing about the

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[1] *Part of this Chapter has been been published in Wride M A and Sanders E J. (1995). Potential roles for tumour necrosis factorα during embryonic development. Anat. Embryol. 191: 1-10.*

observed tumour remission? It was not until 1975 that the latter was shown to be true when the metabolic derangements, associated with infection, were shown to be mediated largely by a range of proteins called cytokines, which are produced by the immune system and alter host metabolism. One such protein was identified, in the serum of endotoxin treated animals, that elicited haemorrhagic necrosis of tumours. The protein was therefore named tumour necrosis factor (TNF) and subsequently TNFα, following the identification of the related molecule lymphotoxin (LT) or TNFß. At the same time, in the search for an explanation for the mechanism of cachexia (a wasting syndrome seen in many chronic disease states) a host factor, produced by macrophages, was isolated and named cachectin. Subsequent analysis revealed that TNFα and cachectin were identical in DNA sequence and that purified cachectin possessed potent tumour necrosis activity. Thus, both cachectin and TNFα bioactivities are due to a single highly conserved protein (Beutler and Cerami, 1986).

2.1.2. The TNF ligand and receptor family

TNFα and its receptors are members of emerging families of type I and II plasma membrane proteins (Bazan, 1993; Smith *et al.*, 1994), which have important roles to play in many aspects of physiology and pathophysiology. The motif that defines the TNF receptor (TNFR) family is that of a four-fold repeat of approximately 40 amino acids, each containing repeats of 6 cysteines (Bazan, 1993; Smith *et al.*, 1994). This motif has also recently been shown to be present in the insulin and epidermal growth factor (EGF) receptors (Ward *et al.*, 1995) and it is confined entirely to the extracellular region in all TNFR family members (Smith *et al.*, 1994). The TNF ligand family members form trimeric complexes like those initially described for TNFα (Peitsch and Tschopp, 1995).

This family of ligands and receptors is summarised in Table 2-1 and, in addition to TNFα, lymphotoxinα (LTα) and their receptors, it includes: the Fas/APO antigen (Leithäuser *et al.*, 1993; Cory, 1994; Lynch, 1995) and its ligand (Suda *et al.*, 1993; Suda and Nagata, 1994); LTβ (Browning *et al.*, 1993) and its receptor (Crowe *et al.*, 1994); CD27 (Camerini *et al.*, 1991; Gravestein *et al.*, 1993) and its ligand CD70 (Goodwin *et al.*, 1993a; Hintzen *et al.*, 1994a; 1994b); CD30 and its ligand (Smith *et al.*, 1993); CD40 and its ligand (Armitage *et al.*,

1992; Spriggs *et al.*, 1993); the p75 NGFR (Rabizadeh *et al.*, 1993); the OX40 ligand (Godfrey, 1994); and the product of the inducible T-cell gene 4-1BB and its ligand (Goodwin *et al.*, 1993b).

There is a large degree of functional overlap between the members of the TNFR and ligand families and receptor-ligand interaction results in cell death, survival, proliferation or differentiation (Peitsch and Tschopp, 1995; Smith *et al.*, 1994). For example, Fas, like TNFα, is capable of instigating cell death by apoptosis (see Chapter 3), while the ligands for CD27, and 4-1BB stimulate cell proliferation and differentiation, and CD40 and p75 NGFR protect cells from death. These ligands and receptors are likely to have evolved through a process of parallel, divergent evolution (Beutler and van Huffel, 1994a) of putative ancestral TNFR and ligand genes, and it is this that may have brought about the diversity, in the cytoplasmic domain structure, that contributes to the wide range of cellular responses attributed to TNF family ligands.

2.1.3. Production and regulation of TNFα

TNFα is a 17kDa protein, produced mainly by monocytes and macrophages, which is active as a 51kDa trimer formed by the association and folding of TNFα monomers (Hlodan and Pain, 1995). Bioactive, oligomeric TNFα dissociates when its concentration is in the picomolar range, while reassociation of its subunits occurs in the nanomolar range (Corti *et al.*, 1992). Drugs, such as suramin, that bind to the TNFα trimer, destabilise it and promote deoligomerisation, leading to inhibition of receptor binding (Alzani *et al.*, 1993; 1995).

The TNFα gene is positioned on the short arm of human chromosome 6 and murine chromosome 17 at the boundary of the major histocompatibility complex (MHC) regions I and III (Vilcek and Lee, 1991). The majority of mammalian cells are not capable of producing TNFα and this is thought to be due to the fact that, in most cells, the TNFα gene is highly methylated and exists within heterochromatin (Beutler, 1995). However, in cells in which the TNFα gene is active, it is up-regulated by several putative 5' sites, which bind transcription factors such as NFκB, C/EBPβ, or AP-2 (Pope *et al.*, 1994; Rhoades *et al.*, 1995), which are induced, for example, during lipopolysaccharide (LPS) activation of macrophages (Ruddle,

1992) or by cytokines, such as granulocyte macrophage colony-stimulating factor (GM-CSF) or interleukin1-β (IL-1β; Bethea *et al.*, 1992; Rhoades *et al.*, 1995). The TNFα gene is also subject to down-regulation through a repressor site (RS) that binds NFκB (Fong and Mark, 1995) and several RS binding proteins, which have yet to be fully characterised (Fong *et al.*, 1995). In addition, TNFα is subject to translational control (Vilcek and Lee, 1991) through a UA-rich sequence at the 3' untranslated region of the TNFα mRNA, which acts to regulate mRNA stability.

TNFα is initially produced as a 26kDa transmembrane protein (Kriegler *et al.*, 1988), which is cleaved by several matrix metalloproteases to release the 17kDa form (Gearing *et al.*, 1995). These authors pointed out that different forms of the TNFα molecule may induce different physiological responses and it has also been demonstrated that processing can result in the production of a larger, inactive form of secreted protein of unknown function (Cseh and Beutler, 1989). Sherry *et al.* (1990) characterised a number of high molecular weight forms of murine TNFα. These isoforms were shown to be due to differential glycosylation at sites on the mature protein and it is possible that differential glycosylation might also affect TNFα activities.

2.1.4. TNF receptors

i. Production and regulation of TNF receptors: TNFα exerts its biological effects through two types of receptors that are responsible for its pleiotropic actions and which were initially identified on human cell lines, using two sets of monoclonal antibodies prepared against TNF binding proteins (Brockhaus *et al.*, 1990). TNF-receptor 1 (TNFR1) is approximately 55kDa in size, while TNF-receptor 2 (TNFR2) is approximately 75kDa in size (reviewed by Rothe *et al.*, 1992; Smith and Baglioni, 1992).

Human TNFRs have been cloned and sequenced (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990; Fuchs *et al.*, 1992). The mouse TNFRs have also been cloned (Goodwin *et al.*, 1991; Lewis *et al.*, 1991) and they share 65% sequence identity, at the amino acid level, to the human TNFRs. These receptors are also capable of binding LTα, although there are quantitative and qualitative differences between the signalling cascades initiated by

TNFα and LTα binding to each type of receptor (Chaturvedi *et al.*, 1994). Both receptors bind TNFα and LTα with K_d values in the picomolar range and they are expressed in varying relative amounts on different cell types, but usually within the range of a few hundred to a few thousand receptors per cell (Roth *et al.*, 1992).

The production of each receptor subtype is regulated independently at both transcriptional and post transcriptional levels (Lindvall *et al.*, 1993). Up-regulation of TNFRs may be a result of the effects of IFN-γ, IL-1α, IL-2, thyroid stimulating hormone, or lectins (Tsujimoto and Oku, 1992; Pandita *et al.*, 1992; Caldwell and Emerson, 1995) and down-regulation is dependent upon factors such as glucocorticoids, phorbol esters, IL-1, and LPS, while TNFα itself may up- or down-regulate its receptors depending on the cell type (Tsujimoto and Oku, 1992). Recently, retinoic acid (RA) has been shown to inhibit TNFα-mediated cytotoxicity (Hughes and Fulep, 1995) and this could be a result of down-regulation of both types of TNFRs (Totpal *et al.*, 1995).

An additional receptor subtype has also been identified, which is specific for TNFα (Schwalb *et al.*, 1993) and it was suggested, by these authors, that post transcriptional modification, or alternative mRNA splicing, may account for the appearance of this receptor sub-type in some cells.

ii. TNF receptor-TNFα interaction, aggregation, internalisation, and shedding: TNFR signalling can be elicited either by association of free homotrimeric TNFα with its receptors or by receptor interaction with plasma membrane-associated TNFα, which itself may be bound to receptors on adjacent cells (Bakouche *et al.*, 1988). Furthermore, each TNFR has a ligand binding domain with a different three-dimensional structure (Chen *et al.*, 1995).

Upon receptor binding, homotrimeric TNFα induces aggregation of the intracellular domains of TNFRs, which contributes towards the transduction of TNFα-specific signalling pathways, such as cytotoxicity (Tartaglia and Goeddel, 1992a,b; Song *et al.*, 1994). In addition, there is evidence that TNFR dimerisation is sufficient to bring about TNFα-specific responses (Adam *et al.*, 1995). Aggregation may be accompanied by the formation of covalent

bonds between the components of receptor clusters (Grazioli *et al.*, 1994). Only homomultimerisation occurs during TNFR clustering and heteromultimerisation, between TNFR1 and TNFR2, is not a significant event in TNFα signal transduction (Moosmayer *et al.*, 1994). Moreover, a significant amount of self aggregation can occur in the absence of TNFα and the same applies for Fas (Boldin *et al.*, 1995a), suggesting that there must be mechanisms in place that restrict the association of receptors, thereby preventing constitutive signalling. One proposed mechanism could involve the dimerisation of the extracellular domains of TNFRs in the absence of TNFα, such that the intracellular signalling domains are physically separated (Naismith *et al.*, 1995).

Finally, an additional consequence of TNFR binding by TNFα is receptor down-regulation. This can occur by internalisation of TNFR1 and shedding of TNFR2 and the signalling for both processes is mediated through TNFR1 (Higuchi and Aggarwal, 1994a; Porteu and Hieblot, 1994). However, it is apparent that TNFR1 can also be shed from cells to form soluble TNFRs (see below).

iii. TNF receptor-mediated responses: The two different types of TNFR can mediate distinct cellular responses (Tartaglia *et al.*, 1991), mainly due to the lack of similarity between their intracellular domains. The degree to which each of the TNFRs contributes to TNFα- mediated effects such as cell death, proliferation, or differentiation is controversial.

The contribution of each receptor subtype to the regulation of cell death is a good example of this controversy. Direct evidence for a role for TNFR2 in cytotoxicity was provided by [illegible] *et al.* (1992) and Higuchi and Aggarwal (1993), while Tartaglia *et al.* (1993a) argued that the role of TNFR2 is primarily to recruit TNFα for TNFR1, which then mediates cytotoxicity. They termed this phenomenon "ligand passing" and argued that TNFR2 acts by increasing the local concentration of TNFα, thereby increasing the amount available to TNFR1. Later, Tartaglia (1993b) conceded that it is possible that TNFR2-mediated signals might promote cell death in some cell types, but they maintained that TNFR1 is the receptor that directly signals cytotoxicity in most cell types and that there was no good evidence for a

similar direct signalling role for TNFR2 in cytotoxicity. In fact, it was suggested that TNFR2 was more likely to have a direct role in the stimulation of cell proliferation (Tartaglia *et al.*, 1993c). However, at this time, there was insufficient evidence to rule out a role for TNFR2 in cytotoxicity (Heller *et al.*, 1993) .

Several, more recent, reports have confirmed that TNFR2 has a role in cytotoxicity. Both types of TNFR are capable of independently mediating cytotoxicity, through protein kinases (PKs), while signalling through TNFR2 occurs primarily through arachidonate metabolism (Grell *et al.*, 1993; 1994a; see below). In the mouse hybridoma cell line PC60, transfected with TNFRs, either TNFR1 or TNFR2 is required for induction of the transcriptional activator NFκB (see below), or cytokines, but apoptosis is only observed in cells containing both receptor sub-types (Vandanabeele *et al.*, 1994). Furthermore, functional co-operation is required, between liganded and non-liganded receptors, for the induction of cell death (Vandanabeele *et al.*, 1995). Further support for this concept of TNFR co-operation was provided by Higuchi and Aggarwal (1994b) who showed that TNFR1 and TNFR2 had differential effects on cytotoxicity, DNA fragmentation, and differentiation in a leukaemia cell line and by Medvedev *et al.* (1994) and Bigda *et al.* (1994) who both showed that TNFR2 induces cytotoxicity in a cell-specific manner and potentiates the effects of TNFR1 on gene regulation and cell death. Finally, the response of individual TNFRs, to ligand binding, may depend on the nature of the TNFα ligand involved, since the transmembrane form of TNFα has been shown to be the prime activator of TNFR2 signalling (Grell *et al.*, 1995).

iv. The "death domain": Tartaglia *et al.* (1993d) have identified a domain within TNFR1 that signals cell death and which is homologous to the intracellular domain of the Fas antigen (Itoh and Nagata, 1993) and the p75 NGFR (Chapman, 1995). Interestingly, this "death domain" is homologous to the recently identified protein reaper, which is involved in programmed cell death (apoptosis) during *Drosophila* embryogenesis (Golstein *et al.*, 1995; see Chapter 3). The "death domains", in TNFR family members, have their effect by self aggregation, as described above. Despite the similarity in the "death domains" of TNFRs and the Fas ligand, the TNFR and Fas "death domains" do not appear to be able to interact with

each other and appear to utilise distinct signalling pathways (Grell *et al.*, 1994b; Schulze-Osthoff *et al.*, 1994; Wong and Goeddel, 1994).

v. TNF receptor associated factors (TRAFs): Recently, several proteins have been identified that interact with the intracellular domains of both Fas and TNFR1, which could be involved in the signalling of cell death (Cleveland and Ihle, 1995). Because Fas and TNFα-induced cell death is initiated within two hours and it is not dependent upon macromolecular synthesis, these proteins could represent some of the latent cytoplasmic cell death effector molecules, which it is hypothesised, must be involved in this process. A protein has been identified, which is related to a proteosomal subunit and binds to TNFR1 just up-stream of its "death domain" (Boldin *et al.*, 1995b). Hsu *et al.* (1995) identified TRADD, a molecule that specifically interacts with the "death domain" of TNFR1 to induce both cell death and NFκB activation. Interestingly, these two pathways are distinct, since the product of the cell death inhibiting poxvirus gene *crmA*, which acts by inhibiting interleukin-1β-converting enzyme (ICE) inhibits cell death induced both by Fas and TNFR1 (Tewari and Dixit, 1995), and is effective in preventing cell death, but not in preventing the activation of NFκB. Chinnaiyan *et al.* (1995) and Boldin *et al.* (1995c) have identified another protein, FADD or MORT1, which interacts with the "death domain" of Fas to initiate apoptosis and this too is inhibited by *crmA*. Finally, RIP (Stanger *et al.*, 1995) is able to associate with Fas, and to a lesser extent with TNFR1, to promote cell death.

In addition to these TRAFs, which are involved in cell death, several other factors have been identified, the precise roles of which are less clearly delineated. TNFRs themselves do not exhibit any PK activity, but Darnay *et al.* (1994) have identified a PK activity (TRAK) that is specifically associated with the TNFR1 cytoplasmic domain and which causes the phosphorylation of a 52kDa protein that is associated with it (TRAP). TRAK interacts with the cytoplasmic domain of TNFR1 in the "death domain" needed for the cytotoxic signalling of TNFα (Darnay *et al.*, 1995). Song *et al.* (1995) have identified proteins (TRAP-1 and TRAP-2) that associate with TNFR1 outside the "death domain". TRAP-1 has homology to heat shock protein 90 (HSP90). These authors point out that the "death domain" is not the only

one involved in TNFR-mediated cell signalling and suggest that different domains within TNFR1 could control important and distinguishable second messenger pathways and mediate distinct cellular responses, through interaction with different TRAFs. Finally, several proteins have been identified that interact with TNFR2. TRAF-1 and TRAF-2 form heterodimers and interact with the cytoplasmic domain of TNFR2 through TRAF-1 (Rothe *et al.*, 1994). These factors are related to CRAF-1, a protein that interacts with the CD40 cytoplasmic domain (Cheng *et al.*, 1995) and to TRAF-3, which can also interact with CD40 and appears to be involved in signalling by activating NFκB (Rothe *et al.*, 1995). Thus, there is an abundance of emerging evidence that is providing important clues about how members of the TNFR family signal downstream cellular responses, via associated proteins.

vi. **Soluble TNF receptors:** Naturally occurring TNFα inhibitors are formed as a result of the release of the extracellular domains of the two TNFRs by proteolytic cleavage. The product released from TNFR1 is approximately 30kDa in size (Olsson *et al.*, 1993), while that released from TNFR2 is approximately 40kDa (Crowe *et al.*, 1993), although differences in the exact molecular weights observed may be due to differential N- and O-glycosylation (Corti *et al.*, 1995a,b). These soluble receptors bind to free monomeric TNFα, thus reducing the amounts available to form trimeric TNFα and, therefore, the amount of trimeric TNFα available to interact with cell surface receptors. By this mechanism, soluble receptors have a number of important roles to play in controlling the effects of TNFα in malignancy, autoimmunity and inflammation (Aderka *et al.*, 1992; Olsson *et al.*, 1993; Dayer and Burger, 1994; van Tits *et al.*, 1994).

Proteolytic cleavage in both TNFRs requires metallo- and serine-proteases (Brakebusch *et al.*, 1994; Björnberg *et al.*, 1995) and occurs at an Asn/Val cleavage site in TNFR1 to produce soluble TNFR1 (sTNFR1; Gullberg *et al.*, 1992). Similarly, sTNFR2 production occurs by proteolytic cleavage, which can be inhibited by a metalloprotease inhibitor (Crowe *et al.*, 1995), and it was demonstrated that phosphorylation of proteins, other than the receptor, was essential for cleavage (Crowe *et al.*, 1993). Indeed, generation of soluble forms of TNFRs can be regulated by PK-A and PK-C (Björnberg *et al.*, 1994).

The release of sTNFRs is influenced by the profile of other cytokines in the cellular environment. Martel-Pelletier *et al.* (1995) demonstrated that transforming growth factor β (TGFβ) was effective in inhibiting release of both soluble receptors, while the addition of IL-1β, in concert with TGFβ, produced the greatest amount of inhibition of release. IL-1 and TNFα enhanced the levels of sTNFR2 compared to sTNFR1 and addition of TNFα and IL-1β produced an inhibition in the release of sTNFR1, while sTNFR2 levels were unaffected. Platelet derived growth factor (PDGF)-BB and insulin-like growth factor (IGF)-1 both enhanced levels of sTNFR1 in a dose-dependent manner, although this could be secondary to an influence of these growth factors on mitogenesis. IFN-α has potent anti-inflammatory properties and it has been shown that this effect is mediated not only by its effect on reducing TNFα synthesis, but also by enhancing sTNFR production (Tilg *et al.*, 1995).

Soluble members of the TNFR family may also be important in the regulation of cell death in tumour cells. Soluble Fas was shown to be present in a human osteosarcoma cell line, which is resistant to the apoptotic effects of Fas (Owen-Schaub *et al.*, 1995) and soluble Fas has been shown to be effective in blocking Fas mediated apoptosis (Cheng *et al.*, 1994). Interestingly, it has also been revealed that the product of the cowpox virus gene, *crmB*, is homologous to sTNFR2 (Hu *et al.*, 1994), an observation that suggests that viruses are capable of modifying TNFα-mediated anti-viral processes, such as apoptosis, by neutralising the functional activity of TNFα.

2.2. Pathways of TNFα signal transduction

Following binding to its receptors and receptor multimerisation, TNFα stimulates a number of cell signalling pathways (Schütze *et al.*, 1992; Heller and Krönke, 1994; Warzocha *et al.*, 1995). These include activation of phospholipase A_2 (PLA_2), which results in the release of arachidonic acid (AA) and metabolites including leukotrienes (LKTs), prostaglandins (PGs), and 5-hydroperoxyeicosatetraenoic acid (5-HPETE); phosphatidylcholine-specific phospholipase C (PC-PLC) and diacylglycerol (DAG) production, resulting in the activation of protein kinase C (PK-C); phospholipase D (PL-D) activation; the hydrolysis of sphingomyelin (SM) to ceramide by neutral and acidic SMases; AP-1 formation from the rapid induction of

interferon-regulatory-factors 1 and 2 (IRF-1 and IRF-2), due to the effects of the nuclear transcription factor NFκB, which is the only direct target of TNFR activation so far identified. In addition, there are likely to be TNFα-specific effects that are mediated by TNFα-induced factors, which have yet to be fully characterised (Lee *et al.*, 1990; Klefstrom *et al.*, 1993). A summary of these pathways, including those for the LT β receptor, is presented in Fig. 2-1.

2.2.1. Second messenger production

Many of the key second messenger pathways, described above, are activated following the binding of TNFα to TNFR1 and it is for this reason that it has been considered that TNFR1 may control the majority of TNFα actions (Wiegmann *et al.*, 1992). However, more recent studies have confirmed that TNFR2 can also contribute significantly to TNFα-induced second messenger cascades.

i. Phospholipase A_2 activation: Activation of PLA_2 results in release of AA, which contributes towards the formation of PGs, LKTs, and eicosanoids and activates PK-C (see Fig 2-1). This pathway is primarily responsible for the pro-inflammatory actions of TNFα, through PGs and LKTs, while eicosanoids, such as 5-HPETE, are likely to be involved in the generation of oxygen free radicals that may be involved in cytotoxicity and the induction of proteins such as manganous superoxide dismutase (MnSOD; see below) that protect cells against the cytotoxic effects of TNFα (Chang *et al.*, 1992; Wong *et al.*, 1992). Furthermore, products of PLA_2 may have direct effects on the activation of oncogenes, such as *c-fos* (Warzocha *et al.*, 1995).

Cytoplasmic Ca^{2+}-dependent PLA_2 ($cPLA_2$) is also an important mediator of the pro-inflammatory actions of TNFα and both its synthesis and activity are enhanced by TNFα binding to its receptors, and, in addition, its activity is enhanced by high Ca^{2+} concentrations in the cell (Hoeck *et al.*, 1993).

PLA_2 appears to be involved in TNFα-mediated cell cytotoxicity in some, but not all cell lines. Robaye and Dumont (1992) were unable to correlate the extent of apoptosis in bovine aortic endothelial cells with changes in PLA_2 activity or to the amount of AA released. However, Jäättelä *et al.* (1992a) and Jäättelä (1993) have shown that HSP 70, itself induced by TNFα, inhibits TNFα-mediated cell death and this is achieved by interfering with the PLA_2 pathway, suggesting that PLA_2 is an essential component of the cell death pathway. In addition, Jäättelä *et al.* (1995) have shown that PLA_2 inhibitors can inhibit Fas- and TNFα-mediated apoptosis in breast carcinoma cells. Furthermore, this cytotoxicity could be reduced by the transfection of these cells with cDNAs encoding for Bcl-2 and Bcl-x, proteins which inhibit cell death, and the effect was specific to PLA_2 metabolism, since NFκB activity was unaffected in these cells.

ii. Phospholipase C and D activation: Within a few minutes of receptor binding, TNFα increases the activity of PC-PLC, which in turn, catalyses the production of DAG from membrane phospholipids without a concomitant increase in Ca^{2+} levels (Heller and Krönke, 1994; Fig. 2-1). DAG then promotes the activation of PK-C, which in turn brings about a multitude of phosphorylation events that ultimately result in alterations in the activities of transcription factors (Warzocha *et al.*, 1995; see below) and possibly in the phosphorylation status of HSPs, such as HSP 27 (Mehlen *et al.*, 1995) and HSP 28 (Vietor and Vilcek, 1994), which protect cells against TNFα-mediated cytotoxicty.

Increased PLD activity is also associated with TNFα cytotoxicity (De Valck *et al.*, 1993). The primary product of PL-D activation is phosphatidic acid (PTDA), which has been shown to be a major mediator of Ca^{2+} mobilisation. Furthermore, PTDA can also be converted to DAG under the influence of PDTA hydrolase. Increased PL-D activity may be due to a TNFα-mediated increase in guanine nucleotide binding protein activity (Klein *et al.*, 1995).

iii. Ceramide: An additional effect of DAG release, due to TNFR activation, is the formation of ceramide, from SM by acidic SMase (see Fig. 2-1). Ceramide is a regulator of apoptosis and growth suppression (Kolesnick and Golde, 1994; Schütze *et al.*,

1994; Hannun and Obeid, 1995; Pushkareva *et al.*, 1995). Activation of acidic SMase, by DAG, occurs in acidic cellular compartments such as lysosomes and endosomes and DAG may be co-internalised with TNFα-TNFR complexes if PC-PLC action has occurred in close proximity to TNFRs in the cell membrane (Heller and Krönke, 1994). Thus, the extent of TNFα–TNFR internalisation in a cell, and the acidity of the compartment in which the complex is present, may have far-reaching consequences for acidic SMase activity and TNFα-mediated cell signalling. Acidic SMase has been shown to trigger NFκB activation, which distinguishes it from neutral SMase (Wiegmann *et al.*, 1994; see below).

Neutral SMase, unlike acidic SMase, is active in the cell membrane (see Fig. 2-1) and is activated by a different cytoplasmic domain, within TNFR1, than acidic SMase (Wiegmann *et al.*, 1994). It has important roles to play in the activation of ceramide activated PKs (CAPKs), raf-kinase, mitogen activated protein kinase (MAPK) and PLA_2 (Kolesnick and Golde, 1994; Belka *et al.*, 1995). As was the case with the PLA_2 pathway, this pathway of TNFα signalling may also activate cellular protective mechanisms against TNFα cytotoxicity, since MAPK can phosphorylate and activate an additional PK (MAPKAP kinase 2) that, in turn, phosphorylates a small HSP (Engel *et al.*, 1995). PK activation may also be important in other examples of TNFα-mediated effects, such as the induction of endothelial cell adhesion molecules (CAMs; Weber *et al.*, 1995).

A recent study has shown that the PLA_2 and neutral SMase pathways interact to modulate the activity of a PK-C isozyme called PK-ζ (Müller *et al.*, 1995). Thus, PK-ζ appears to occupy a central position in the mitogenic and growth inhibiting pathways of TNFα by behaving as a central switch between two major pathways of TNFα signal transduction.

iv. Oxygen free radicals: The generation of reactive intermediates of oxygen metabolism (free radicals) is also an important second messenger pathway in response to TNFα binding by its receptors (see Fig. 2-1). Free radicals, increased by TNFα, include superoxide anions ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) and these may be important in the activation of transcription factors and in TNFα-mediated cytotoxicity (Wong *et al.*, 1992).

Mitochondrial free radical generation may be an important component of the cytotoxic activity of TNFα, since mitochondrial respiratory chain inhibitors inhibit TNFα-mediated cytotoxicity and TNFα results in damage to mitochondrial chain components, as judged by morphological criteria (Shulze-Osthoff *et al.*, 1992). In addition, mitochondria-derived free radicals are transducers of TNFα-mediated gene expression through activation of the transcription factor NFκB and depletion of mitochondrial electron transport results in abrogation of both the cytotoxic and gene-inductive effects of TNFα (Schulze-Osthoff *et al.*, 1993). Furthermore, a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase has been shown to be involved in the TNFα-mediated induction of free radicals that influence induction of genes, such as colony stimulating factor-1 (CSF-1), and monocyte chemoattractant protein (Satriano *et al.*, 1993).

In view of the influence of TNFα on free radical production by mitochondria, and subsequent NFκB activation, it is interesting that this pathway may be involved in the up-regulation of ferritin (Kwak *et al.*, 1995), a protein that protects against oxidative damage by chelating iron, which is involved in free radical formation and TNFα-mediated cytotoxicity (Warren *et al.*, 1993). Furthermore, it has been demonstrated that copper contributes to TNFα-mediated cytotoxicity via TNFR1 and this pathway is independent of PLA_2 activation (Wada *et al.*, 1994).

A number of antioxidants, proto-oncogenes, and viral proteins are effective in combating TNFα-mediated free radical damage, including metallothionein, MnSOD, *N*-acetylcysteine, crmA, and Bcl-2 (Sciavolino, 1992; Wong *et al.*, 1992; Leyshon-Sørland *et al.*, 1993; M. Sato *et al.*, 1995; Talley *et al.*, 1995). Interestingly, TNFα can protect against TNFα-mediated cytotoxicity, when expressed in sensitive cells (Vanhaesebroeck *et al.*, 1992), and cells that produce TNFα, but which are resistant to its cytotoxic effects, have been shown to achieve this resistance by upregulation of MnSOD synthesis (Okamoto *et al.*, 1992). In addition, the extent of TNFα-mediated cytotoxicity may be intimately influenced by the oxygen content of the cellular microenvironment (Sampson and Chaplin, 1994).

However, not all TNFR family members, which are capable of mediating cytotoxicity, may require reactive oxygen intermediates for their effects, since Fas-mediated apoptosis can occur in the presence of antioxidants (Hug *et al.*, 1994).

2.2.2. Transcription factors

i. NFκB: Transcription factors represent the last link in the chain between the cell membrane and the nucleus and the most important transcription factor in TNFα signal transduction, is NFκB (Worzocha *et al.*, 1995). Activation of NFκB by TNFα occurs within 10-15 minutes and, although TNFRs are required for the transduction of the TNFα-mediated signal, activation requires only 10-25% of total receptor occupancy by TNFα (Chan and Aggarwal, 1994). There appear to be multiple TNFα-inducible NFκB complexes, including the p50, p65, c-rel, and p52 oncoproteins, that contain unique combinations of NFκB family members, suggesting that NFκB responsive genes may be regulated in a subunit specific manner (Beg and Baldwin, 1994). NFκB is normally associated with an inhibitory protein, IkB-α, and the complex is sequestered in the cytosol. Activation of NFκB occurs by post translational means and results from the dissociation of the NFkB• IkB- α complex and the subsequent translocation of NFκB to the nucleus (see Fig. 2-1). This dissociation is a result of TNFα- or okadaic acid-induced activation of a protein-tyrosine phosphatase, which activates a downstream PK leading to phosphorylation of a serine residue within IkB-α, which leads to its degradation (Menon *et al.*, 1995).

Although both types of TNFR can activate NFκB, it has been shown that TNFR2 is capable of NFκB activation independently and this occurs 15 minutes later than activation of NFκB by TNFR1 and requires the cytoplasmic domain of TNFR2 (Lægrid *et al.*, 1994; Rao *et al.*, 1995).

Reddy *et al.* (1994) showed, using a cell reconstitution system consisting of membrane, cytosolic, and nuclear fractions from un-stimulated cells, that TNFα-mediated NFκB activation requires membrane associated components, which were identified as belonging to the DAG (Yang *et al.*, 1993) and ceramide pathways (Dbaibo *et al.*, 1993). However, although the DAG and ceramide pathways are involved in TNFα-mediated activation of NFκB, they are not

likely to be the only ones, since induction of NFκB by TNFα was not completely abrogated by phorbol 12-myristate (PMA), a drug that inhibits these pathways (Johns *et al.*, 1994) .

ii. AP-1: An important mediator of the mitogenic effects of TNFα on cells appears to be AP-1, which is formed by dimerisation of c-fos and c-jun proteins (see Fig. 2-1). Brach *et al.* (1993) demonstrated that antisense oligonucleotides to *c-fos* inhibited the formation of AP-1 and the proliferative response of human fibroblasts to TNFα. In addition, AP-1, along with an additional factor called PEA3, is involved in the induction of urokinase/plasminogen activator, an important enzyme in the control of tissue remodelling (Lengyel *et al.*, 1995) and AP-1 is also involved in the down-regulation of elastin gene expression (Kähäri et al., 1992).

iii. c-myc: The cytotoxic effects of TNFα appear to be mediated, at least in part, by c-myc protein. TNFα-sensitive cells undergo a rapid increase in the amount of nuclear c-myc, following the addition of TNFα, compared to cell lines that are resistant to the cytotoxic effects of TNFα (Jänicke *et al.*, 1994). This effect was not dependent upon rapid *de novo* protein synthesis following TNFα addition, since it occurred in the presence of cycloheximide, a protein synthesis inhibitor. However, the cytotoxic effect of TNFα could be inhibited in sensitive cells by the addition to them of an antisense *c-myc* oligonucleotide 6 hours before the addition of TNFα. This result suggests that *c-myc*-dependent cytotoxicity is a result of the activation of existing c-myc protein synthesised before the addition of TNFα to the cells. Similar observations were reported by Klefstrom *et al.* (1994) who also showed that c-myc and TNFα-induced cell death are inhibited by expression of the bcl-2 oncoprotein and the free radical scavenging enzyme MnSOD.

iv. Transcription factor co-operation: Other cytokines may act in concert with, or as antagonists of, TNFα through overlapping pathways that regulate the binding of transcription factors to responsive elements. For example, TNFα and TGFβ have opposing effects on collagen gene transcription; TGFβ enhances, while TNFα inhibits this process. This

has been shown to be due to the binding of a protein complex, induced by TNFα, to a negatively acting element adjacent to a positively acting element that is activated by TGFβ (Inagaki *et al.*, 1995). On the other hand, NFκB is an important component of the signalling pathway leading to the induction of endothelial CAMs by TNFα (Takeuchi and Baichwal, 1995) and this can be enhanced by other factors, such as IFNγ (Jahnke and Johnson, 1994; 1995).

2.2.3. TNFα signalling without receptors

Both TNFα and LTα may be able to mediate cell signalling directly at the cell membrane through their ability to form membrane channels, at low pH, as a result of their trimeric structure (Kagan *et al.*, 1992; 1993). This activity may contribute to cytotoxicity via direct membrane damage or through passage of ions such as K^+, Mg^{2+}, or Ca^{2+} out of, and into, the cell through the channel.

2.3. Effects of TNF ligand and receptor family mutations

Attempts have been made to decipher functions within the TNFR and ligand families by noting the effects of naturally occurring and man-made mutations (Beutler, 1995; Beutler and van Huffel, 1995b). A summary of mutations to, and knockouts of, various members of the TNF ligand and receptor family is presented in Table 2-1.

Naturally occurring mutations to the Fas antigen, and its ligand, result in lymphoproliferative disease, possibly due to a failure in apoptosis (Takahashi *et al.*, 1994), while mutations in the CD40 ligand result in faulty T-cell dependent activation of B-cells, such that there are alterations in the profile of the immunoglobulins produced (Allen *et al.*, 1993; Korthäuer, *et al.*, 1993). Furthermore, targeted mutations to the p75 NGFR result in aberrant sensory innervation of peripheral structures (Lee *et al.*, 1992).

No naturally occurring mutations of TNFα, LTα, or their receptors have been identified (Beutler, 1995). Therefore, it has been necessary to introduce these mutations into mice using gene targeting approaches (Rossant and Hopkins, 1992), and studies in which

TNFRs have been "knocked out", or in which a constitutive inhibitor for TNFα, LTα, or LTβ has been introduced into mice, have provided useful information about TNFR functions.

Mice that lack TNFR1 have been genetically engineered (Pfeffer *et al.*, 1993; Rothe *et al.*, 1993). These mice display normal numbers of TNFR2, but the mutation renders them resistant to lethal doses of LPS or *S. aureus* enterotoxin B, and, in contrast, they are highly sensitive to *L. monocytogenes* infection. In fibroblasts from TNFR1 deficient mice, the signalling pathways of the two TNFRs were further characterised (Mackay *et al.*, 1994). It was revealed that TNFR1 controls several TNFα-induced functions: up-regulation of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, CD44, and MHC class I; secretion of other cytokines, such as IL-6 and GM-CSF; cell proliferation; and activation of NFκB, while stimulation through TNFR2 had no effect on these functions. A TNFR2 knockout has also been reported, which exhibits a minimal phenotype in which only modest resistance to the lethal effects of TNFα injection is seen and there is a protective effect against the damaging effects of TNFα injected under the skin (Erickson *et al.*, 1994). Thus, in gene knockout experiments, TNFR1, rather than TNFR2, would appear to have the more essential role in the defence of the host against micro-organisms, and their associated pathogenic factors, and would appear to be the predominant receptor involved in the activation of TNFα-mediated cell signalling pathways.

Simultaneous gene knockout of TNFR1 and TNFR2 has now been reported and the effect seen is a combination of the TNFR1 and TNFR2 knockouts (Beutler, 1995). It could be envisaged that this result would be similar to that obtained by the expression of a TNFα, LTα and LTβ inhibitor in transgenic mice (Peppel *et al.*, 1993). Indeed, similar effects, on the ability of these mice to respond to infection, were observed as in TNFR1 knockout mice (Kolls *et al.*, 1994).

The knockout of LTα has also been reported (De Togni *et al.*, 1994). It was revealed that LTα is essential in the development of lymph nodes and Peyer's patches, but not the thymus. The implication from this result, and the results of TNFR knockouts (which gave no developmental anomalies), is that LTα must be able to induce cell signalling, essential to the development of peripheral lymphoid organs, independently of its previously recognised

receptors, TNFR1 and TNFR2. This has, indeed, been shown to be the case, since LTα can form a heteromeric complex with LTß (Browning *et al.*, 1993) that interacts with a LTß-specific receptor (Crowe *et al.*, 1994; see Fig. 2-1).

Deletion of TNFα, in combination with LTα, gave an identical phenotype to that obtained with LTα alone (Beutler, 1995), suggesting that TNFα is not required in peripheral lymphoid organ development. However, these results contrast with those of Kossodo *et al.* (1992) who demonstrated that the injection of anti-TNFα into pregnant mice results in effects on mouse foetal growth and lymphoid tissue development.

Thus, the results of studies in which gene targeting technology has been used to disrupt expression of members of the TNF ligand and receptor families has provided useful information regarding the roles of these molecules in physiological and pathophysiological processes. However, the question of the extent to which TNF ligand and receptor family members have roles in developmental processes has not been adequately resolved, since the experiments have provided conflicting results. This issue will be returned to, and discussed more thoroughly, in Chapter 8, in the context of the studies described in this thesis.

2.4. TNFα in disease

TNFα has had a dual history, which extends back to its initial characterisation as a factor that could induce the haemorraghic necrosis of tumours and act as an endogenous mediator of cachexia during chronic disease states (Beutler, 1995; see above). TNFα has wide ranging roles in many pathophysiological situations, including systemic inflammatory response syndrome (SIRS) during bacterial infections, advanced HIV disease, severe malaria, graft versus host reaction and graft rejection, multiple sclerosis (MS), arthritis, cancer, and cachexia (Vassalli, 1992; Herdegen *et al.*, 1993).

TNFα is instrumental in the maintenance of physiological homeostasis, when produced locally and at relatively low concentrations, and it may contribute towards the regulation of several normal processes, including sleep, appetite, and body temperature (Strieter *et al.*, 1993).

As TNFα concentrations increase, as a result of a local injury, TNFα orchestrates a local inflammatory response and exerts paracrine and autocrine effects on cells in the region of the injury, for example during wound healing (Feiken *et al.*, 1995). Thus, the role of TNFα, in this situation, is to initiate, maintain and resolve local inflammation. However, TNFα can reach high enough levels to escape from the region of the local inflammatory response into the systemic circulation. It is at this point that the effects of TNFα, on the host, become increasingly detrimental as its concentration increases and symptoms associated with disease begin to occur, which include fever, loss of appetite, cachexia, and lethargy (Streiter *et al.*, 1993). Cytokines, including TNFα, can cross the blood-brain barrier and are involved in signalling the brain that infection has occurred, thereby initiating centrally-mediated illness responses such as fever (Watkins *et al.*, 1995).

At even higher concentrations, TNFα can cause death as a result of SIRS, which is accompanied by cell damage and tissue injury (due, in part, to dysregulated free radical production; Lloyd *et al.*, 1993), hypotension, dehydration, myocardial suppression, capillary leakage, and multiple organ failure (Tracey, 1992; Cerami, 1993; Streiter *et al.*, 1993; Tracey and Cerami, 1994).

It is important to point out that TNFα does not act alone in these pathophysiological situations and other factors, such as macrophage-migration inhibitory factor (MIF; Bucala *et al.*, 1994) and IL-1, IL-6, and IL-8 (Cavaillon *et al.*, 1992), are all produced and can enhance and inhibit the production of each other. An example of the differing effects that TNFα might have, when interacting with or without other cytokines, is its profound effect on catabolic and anabolic energy metabolism, which can lead, surprisingly, to either cachexia or obesity (Spiegelman and Hotamisligil, 1993). These authors point out that cachexia tends to be caused by TNFα acting at high concentrations and in concert with other cytokines, while obesity occurs as a result of its expression, at lower levels, in the absence of other cytokines.

The intention here is not to provide an exhaustive review of the effects of TNFα in all aspects of pathophysiology, but rather to provide an overview of some of its roles in these processes, focusing on the more recent literature.

2.4.1. Autoimmune disorders

TNFα is involved in several autoimmune disorders (Raine, 1995; Arend and Dayer, 1995), including arthritis, Crohn's disease, systemic lupus erythematosus, some forms of diabetes, and MS. *In vivo* studies have generally suggested that TNFα has a pathogenic role, while *in vitro* experiments have provided evidence that TNFα is protective in these diseases (Jacob, 1992). MS and arthritis will be focused on here.

i. Multiple sclerosis: Butt and Jenkins (1994) demonstrated that TNFα promotes oligodendrocyte damage and demyelination, resembling the changes occurring during MS, in the mouse optic nerve. Chofflon *et al.* (1992) showed that relapsing-remitting MS patients had a higher concentration of TNFα in their blood, during a relapse, than healthy volunteers and that TNFα was found at abnormally high levels six weeks before patients gave clinical symptoms of a relapse. Furthermore, TNFα mRNA levels, in the circulating mononuclear cells of MS patients, were also measured and found to give an indication of the extent of disease activity (Rieckmann *et al.*, 1995). Finally, the antidepressant drug rolipran, which inhibits TNFα production by auto-reactive T helper cells, has been shown to be effective in the treatment of MS (Raine, 1995).

ii. Arthritis: TNFα also appears to play a dominant role in arthritis. Arthritis is manifested by the degradation of the cartilage matrix by enzymes such as stromelysin-1 and collagenase, which can be induced by the action of TNFα, present in the synovial fluid, on chondrocytes (Jasser *et al.*, 1994). TNFα may also contribute indirectly to the pathogenesis of arthritis by increasing the production of IGF-1 binding proteins, thus reducing the anabolic effect of IGF-1 on cartilage matrix synthesis by chondrocytes (Olney *et al.*, 1995). In addition, sTNFRs may have a beneficial role in the different pathologies associated with rheumatoid arthritis, reactive arthritis, and osteoarthritis, since the profile of sTNFRs is different in the synovial fluid and serum of patients with each of these diseases (Steiner *et al.*, 1995).

2.4.2. Cancer

During tumour growth and metastasis, TNFα may have a number of effects, both detrimental and beneficial. The realisation that TNFα was one of the major proteins induced by Coley's toxins and the ability of TNFα to promote necrosis of solid tumours and to exert cytotoxic and cytostatic effects on cancerous cells *in vitro* prompted a great deal of research on its potential uses in cancer therapy (Männel *et al.*, 1993; Sidhu and Bollon, 1993). However, its use in cancer treatment has been limited, because it is required to be administered in high doses, since it has a short half life in humans. Consequently, this can lead to many of the complicating factors cited above; e.g. SIRS. Also, its effects are probably not optimal when it is administered in isolation, since it requires the presence of other cytokines that add to its anti-cancer effect.

The most effective results, in the treatment of malignancies, have been seen with local administration of TNFα, where the detrimental systemic effects of TNFα are less pronounced (Hieber and Heim, 1994). Other forms of therapy, in the future, might include the use of retroviruses to transfer TNFα cDNA into tumour cells; for example, colon carcinoma cells show reduced growth when transfected in this way (Walther *et al.*, 1993), or the use of new, less toxic forms of Coley's toxins (Mizuno and Soma, 1992).

In addition, TNFα may actually promote tumour progression in several ways. For example, by activating enzyme cascades, such as proteases, leading to enhanced tumour cell metastasis and disease progression; by promoting angiogenesis; and by increasing the adherence of tumour cells to endothelium, through upregulation of CAMs. For example, the number of malignant breast cancer cells, expressing TNFα, increases with increasing tumour grade (Miles *et al.*, 1994) and TNFα is associated with endometrial tumours in which its expression may be correlated with a reduced ability for TNFα producing, tumour infiltrating, host immune cells to destroy the tumour cells (Garcia *et al.*, 1994).

2.4.3. Neurodegenerative disease

TNFα has a number of physiological roles in the development and differentiation of the nervous system (Merrill, 1992; see below), but it has also been shown to be involved in

neurodegenerative conditions, including the damage associated with stroke and head injury (Rothwell and Relton, 1993; Feuerstein *et al.*, 1994). Furthermore, TNFα and TNFRs have been immunocytochemically localised to the brains of Parkinson disease subjects, where they may have a role in the degenerative processes associated with the disease (Boka *et al.*, 1994). On the other hand, there is evidence to suggest that TNFα, and LTα, have a protective role, in the brains of Alzheimer's disease patients, by combating the toxic effects of the amyloid ß-peptide (Barger *et al.*, 1995). This appears to be achieved by NFκB-dependent transcription of antioxidant enzymes that protect against the effects of oxygen free radicals induced by the amyloid-ß protein. Similarly, TNFα has a protective function in the response of the brain to injury by promoting the maintenance of Ca^{2+} homeostasis (Cheng *et al.*, 1994) and by upregulating the expression, by astrocytes, of metalloproteases, such as gelatinase A and B, that contribute to tissue remodelling after brain injury (Gottschall and Yu, 1995).

A greater understanding of the physiological roles of TNFα in the nervous system (see below) may also lead to therapeutic strategies, which may be useful in the treatment of neurodegenerative disease.

2.5. TNFα in the nervous system

2.5.1. TNFα in normal nervous system function

It is thought that the contribution of TNFα to pathological (see above) or physiological processes in the brain may, again, be dependent upon its concentration (Merrill, 1992). The beneficial and harmful effects of the production of TNFα, by foetal microglial cells, following addition of LPS to them, are modulated by other cytokines, such as IL-6, IL-10, and TGFß, which suppress TNFα release and of IL-1, which enhances TNFα release (Chao *et al.*, 1995).

TNFα immunoreactivity has been shown to be widespread within the mouse central nervous system (CNS) being associated with neurons in the hypothalamus, bed nucleus of the stria terminalis, parabrachial nucleus, dorsal vagal complex, nucleus ambiguus, and thoracic sympathetic preganglionic column (Breder *et al.*, 1993). It was concluded, from this study, that TNFα, produced by neurons, might serve as a neuromodulator in CNS pathways involved in the regulation of the autonomic, endocrine, and behavioural components of the acute phase

response to infection and inflammation. Indeed, it is recognised that TNFα is a modulator of hormone and transmitter release in central neurons, and it has been demonstrated recently (Soliven and Wang, 1995) that TNFα can mediate nicotinic responses in cultures of rat sympathetic neurons, via factors secreted by non-neuronal cells. In addition, TNFα has been shown to increase the frequency of spontaneous miniature synaptic currents in cultured rat hippocampal neurons, following its secretion by activated astrocytes, and TNFR1 is also present in these cultures (Grassi *et al.*, 1994). In mollusc neurons, TNFα induces hyperpolarisation, due to an effect on decreased Na^+ conductance and by activation of the Na^+ pump (Mimura *et al.*, 1994), and TNFα, in concert with IFNα or IFNß, can modulate the frequency of miniature end plate potentials at rat neuromuscular junctions (Caratsch *et al.*, 1994).

2.5.2. TNFα in the development of the nervous system

As well as its involvement in pathological processes in the brain and normal brain function (see above), TNFα has a number of roles in brain development, which, as mentioned above, may be a consequence of its expression at relatively low concentrations (Merrill, 1992).

A number of cytokines, including TNFα, have been shown to be expressed in the mouse brain during development (Burns *et al.*, 1993) and it was suggested that they may have roles in developmental processes that resemble regenerative or inflammatory processes or in programmed cell death (see Chapter 3). During oligodendrocyte development, TNFα may have important roles in differentiation or programmed cell death, through modulation of signalling pathways, including sphingomyelin hydrolysis and alteration of ion channel conductivities, which could result in inhibition of the phosphorylation of myelin basic protein leading, in turn, to effects on neuron myelination (Soliven and Szuchet, 1995). TNFα, produced by astrocytes, has been shown to be present during glial differentiation and again the effects of TNFα on this process can be modulated by other cytokines (Mizuno *et al.*, 1994). For example, trophic factors, such as ciliary neurotrophic factor (CNTF), produced by astrocytes, may protect against the effects of TNFα-induced cell death during development and disease (Louis *et al.*, 1993).

2.6. TNFα in immunity

2.6.1. TNFα in the development of the thymus

Several cytokines, including ILs, GM-CSF, and LTα, have been identified, by polymerase chain reaction (PCR), during foetal thymus development (Montgomery and Dallman, 1991); however, in this study, TNFα was not looked for. Deman *et al.* (1992) used *in situ* hybridisation to analyse the pattern of TNFα mRNA expression during mouse thymus development. TNFα mRNA expression began in isolated cells at day 14 of development, disappeared during day 15, became pronounced at day 16 and increased progressively up until day 20. After birth, no TNFα mRNA was detected. Furthermore, no correlation could be found between TNFα mRNA expression and cells of the macrophage lineage. On the other hand, using a chloramphenicol acetyltransferase (CAT) reporter gene construct, which replaces the TNFα gene sequence and introns in the murine genome, it has been demonstrated that TNFα appears to be constitutively expressed in the thymus, but in no other tissues examined (Giroir *et al.*, 1992). CAT activity was associated with both the $CD4^+$ and $CD8^+$ thymocyte populations, but not with thymic macrophages or dendritic cells or splenic thymocytes. In addition, both TNFR1 and TNFR2 have been identified in the thymus (Ryffel and Mihatsch, 1993).

Despite these studies, describing TNFα and TNFR expression during thymus development, there was no information provided as to the potential functions of TNFα in this process, although it had been suggested that TNFα might have effects on thymocyte proliferation or selection, through deletion of self-reactive clones by apoptosis and an influence on the expression of molecules mediating cell-cell contact (Deman *et al.*, 1992; Giroir *et al.*, 1992).

Some insights into the potential roles of TNFα, in thymic ontogeny, were revealed by de Kossodo *et al.* (1992), who injected antibodies to TNFα into pregnant mice and noted that, as a result of this, there was a marked atrophy of the thymus as well as the spleen and lymph nodes. These results suggest a role for TNFα in the early development of lymphoid tissues and in the growth of the thymus and spleen in particular, although they should be interpreted with caution, since the effects observed could be secondary to the effects of anti-TNFα on

reproductive process (see below). In support of this, it has been revealed, using a constitutively expressed TNFα, LTα, and LTß inhibitor in a transgenic mouse line, that mice expressing the inhibitor are normal with respect to the size and morphology of their lymphoid organs (Peppel *et al.*, 1993). Thus, these authors concluded that TNFα may be dispensable during normal thymic development.

However, Probert *et al.* (1993) have described the results of a study in which human TNFα was over-expressed in the T-cell compartment of mice. These mice developed pronounced histological and cellular changes in their lymphoid organs (including the thymus, lymph nodes and spleen), a reduced number of thymocytes and peripheral T-cells, and severe cachexia, which resulted in death. The extent of the abnormalities observed depended on the extent of TNFα expression, since at lower levels of TNFα transgene expression, the lethal wasting effects could be dissociated from the effects on lymphoid organs. This is a very important observation for any consideration of a role for TNFα in development and the localised production of low levels of TNFα, that may have autocrine and paracrine roles during development, is a theme that will be returned to in subsequent Chapters of this thesis. All of the observed effects of TNFα could be abrogated using monoclonal antibodies to human TNFα and, since human TNFα does not interact with mouse TNFR2 (Lewis *et al.*, 1991), all of the effects observed could be attributed to TNFR1; for example, enhancement of TNFα mediated cytotoxicity. TNFR2, on the other hand, could be involved primarily in thymocyte proliferation (Tartaglia *et al.*, 1993c), since agonistic rabbit polyclonal antibodies against mouse TNFR2, but not TNFR1, stimulate this process in thymic and peripheral T-cells.

The intrathymic stage of T-cell development is characterised by high levels of thymocyte death and proliferation (Hernándes-Casselles and Stutman, 1993) and TNFα has been implicated in both apoptosis and proliferation in the thymus. For example, Kizaki *et al.* (1993) showed that TNFα may act in concert with cAMP in immature thymocytes to modulate thymocyte programmed cell death, while Hernández-Caselles and Stutman (1993) showed that TNFα interacts with mitogenic factors, such as IL-6, to promote intrathymic thymocyte proliferation and can augment thymocyte apoptosis. Furthermore, these differential effects of TNFα, in the thymus, appear to be mediated by different epitopes of the TNFα molecule.

Finally, TNFα may also have roles in thymocyte differentiation and lineage commitment. All mouse thymocytes go through a stage during their development where they express CD25 (IL-2 receptor α chain), which is an essential step during thymocyte maturation, and it has been discovered that TNFα, in conjunction with IL-1, has a role in inducing this molecule on thymocyte precursors (Zúñiga-Pflücker, 1995).

2.6.2. TNFα in haematopoiesis

TNFα can potently stimulate haematopoiesis *in vivo* and this is a role consistent with the need for increased haematopoiesis in all blood cell lineages during the response of the host to infection (Ulich *et al.*, 1993). TNFα has bifunctional effects during haematopoiesis *in vitro* as an inhibitor or enhancer of the clonal growth of primitive and committed bone marrow progenitors (Jacobsen *et al.*, 1994a). The specific effect of TNFα depends on the profile of other cytokines present. For example, TNFα behaves as an inhibitor of haematopoiesis in combination with granulocyte colony-stimulating factor (G-CSF), colony stimulating factor-1 (CSF-1), erythropoeitin (Epo), and stem cell factor (SCF), while it enhances the clonal growth of bone marrow progenitors, at low concentrations, either on its own or in combination with GM-CSF or IL-3.

The nature of TNFα-mediated effects may also depend on the extent of differentiation of a particular cell type. TNFα synergises with IL-3 to promote proliferation of a subset of haematopoeitic progenitor cells up until 12 days of culture, but after this, as the progenitor cells began to differentiate into granulocytes, it begins to have inhibitory effects on the proliferation and differentiation of these cells (Mannoni and Banchereau, 1991). This kind of effect of TNFα has been confirmed by Rogers and Berman (1994) who showed that TNFα is capable of promoting the proliferation of multipotential progenitor cells, while at the same time inhibiting the further differentiation of committed cells. Furthermore, these workers suggested that many of the contradictory effects of TNFα, observed on haematopoietic cells *in vitro*, might be due to the presence of cells at various stages of maturity in cultures.

These bifunctional effects of TNFα are mediated by TNFR1 and TNFR2 and it is apparent that differences in which responses are signalled by which TNFR depend on the cell

type and the stage of differentiation of the TNFα-sensitive cells as well as the profile of other cytokines present. In cultures of primitive human haematopoietic cells both TNFR1 and TNFR2 are capable of mediating the inhibition of proliferation of primitive bone marrow progenitor cells by agonistic anti-TNFR1 and anti-TNFR2 antibodies (Rusten *et al.*, 1994a). In the same study, the stimulation of progenitor cell proliferation observed in the presence of IL-3 or GM-CSF and low concentrations of TNFα was an event mediated specifically by TNFR1. Furthermore, in cultures of cells in more advanced stages of differentiation, TNFα-mediated effects, during the stimulation of colony formation by IL-3 or GM-CSF and the inhibition of colony formation by G-CSF, were signalled through TNFR1. In a similar study, it was shown that TNFR1 is the receptor involved during TNFα-mediated inhibition of the stimulation of $CD34^+$ haematopoietic progenitor cell proliferation and colony formation in the presence of SCF and CSF (Rusten *et al.*, 1994b). On the other hand, proliferation and differentiation into macrophages, of $CD34^+$ primitive progenitor cells, is stimulated by TNFα, through TNFR1, in the presence of SCF and IL-7 and this effect is reversed by TGFß (Fahlman *et al.*, 1994).

TNFR2 has been shown to be involved in the TNFα-mediated inhibition of primitive mouse haematopoietic progenitor cell proliferation, while only TNFR1 is involved in the TNFα-mediated inhibition of the proliferation of mature cells, of the same type, responding to IL-3 or G-CSF (Jacobsen *et al.*, 1994b; 1995). Finally, TNFR1 and TNFR2 are also involved in erythropoeisis and TNFα directly and potently inhibits the proliferation of committed erythroid progenitor cells in response to multiple cytokine combinations *in vitro* through TNFR1, though TNFR2 can mediate similar effects on cells responsive to Epo alone (Rusten and Jacobsen, 1995).

Finally, TNFα may also modulate haematopoietic cell proliferation and differentiation via the modulation of cytokine receptor expression. For example, Jacobsen *et al.* (1992) have demonstrated that TNFα inhibits bone marrow progenitor cell proliferation by directly down-regulating receptors for CSF, while the proliferation of these cells is enhanced by TNFα-mediated GM-CSF receptor and IL-3 receptor up-regulation.

2.6.3. TNF ligand and receptor family members in T- and B-cell activation

Several members of the TNF ligand and receptor family have important roles in contact mediated B-cell-T-cell collaboration, including: CD27 and its ligand CD70 (Lens *et al.*, 1995); CD40 and its ligand (Spriggs *et al.*, 1993); CD30 and its ligand (Smith *et al.*, 1993); 4-1BB and its ligand (DeBenedette *et al.*, 1995); OX-40 and its ligand gp34 (Godfrey *et al.*, 1994); TNFα and its receptors (Ware *et al.*, 1991); and Fas and its ligand (Alderson *et al.*, 1995). These ligands have differential capacities to influence B-cell function. For example, the CD40 ligand, expressed on activated T-cells, upregulates many functional activities of B-cells including proliferation, cell-cell adhesion, and transcription of immunoglobulin (Ig) constant regions (Spriggs *et al.*, 1993), while CD30 exerts opposing effects on B-cell function (Jumper *et al.*, 1995) and Fas, and its ligand, are involved in activation-induced death in human T-lymphocytes (Alderson *et al.* 1995).

As is the case with TNFα, soluble forms of TNF family ligands and receptors are also important in T- and B-cell mediated immunity. For example, in addition to the membrane-bound form, a soluble form of the CD40 ligand is released as a result of proteolytic processing of full-length CD40 ligand in an intracellular compartment within activated T-cells (Graf *et al.*, 1995) and this too has a role in B-cell activation. In addition, CD27 is capable of forming a soluble receptor as a result of proteolytic processing, on activated B- and T-cells, in a similar process to that described earlier for the TNFRs (Loenen *et al.*, 1992).

Transcription of the TNFα gene is one of the earliest events that occurs following T- or B-cell activation by their antigen receptors (Goldfeld *et al.*, 1992). Following CD40 or IL-4 activation of B-cells, TNFα is released and has an autocrine effect on B-cell proliferation (Boussiotis *et al.*, 1994). The role of TNFα in cell-mediated immunity has been investigated by the administration of anti-TNFα antibodies to mice (Bromberg *et al.*, 1992). It was demonstrated that TNFα appears to be important during the initial priming events in immunity and, therefore, anti-TNFα could alter the early phase of the immune response, but inhibition of TNFα effects later in the response was ineffective, probably because of the presence of multiple other cytokine pathways that can bypass TNFα.

The 26kDa transmembrane form of TNFα, expressed on $CD4^+$ T-cells, is important in providing a co-stimulatory signal for human B-cell activation by IL-4 (Aversa *et al.*, 1993). Membrane-bound TNFα was induced within 2 hours of stimulation with concanavalin A and peak expression was observed after 24 hrs. Antibodies to membrane-bound TNFα inhibited the synthesis of IgM, IgG, IgG4, and IgE. Similarly, during HIV infection, the CD40 ligand is lacking on HIV-infected T-cell clones, but polyclonal B-cell activation can still occur, to a certain extent, and this is due to the expression of membrane-bound TNFα on some T-cells (Macchia *et al.*, 1993).

TNFR regulation is also influenced by B-cell activation. Erikstein *et al.* (1991) have shown that resting B-cells express low amounts of TNFR2 and this receptor is markedly upregulated following activation, whereas TNFR1 levels are low on both activated and resting B-cells. Furthermore, neither TNFR could be upregulated by the addition of ILs 1-8 IFN-γ, or TNFα and LTα.

2.7. TNFα in reproduction

There is an abundance of evidence implicating TNFα in autocrine and paracrine processes that are central to the physiology and pathophysiology of reproduction (Ben-Rafael and Orvieto, 1992; Haimovici and Anderson, 1993; Hunt, 1993; Terranova *et al.*, 1995). TNFα may have important roles to play in gamete and follicle development, steroidogenesis, cancers of the female reproductive tract, uterine cycling, placental differentiation, embryo implantation, embryonic development, and parturition. For example, Hunt *et al.* (1993) carried out a detailed study of the expression patterns of TNFα in several different strains of mice and found it expressed in the oviduct, uterine epithelial cells, decidual cells, macrophage-like cells, placental trophoblasts, and embryos.

2.7.1. TNFα in the ovary

TNFα gene expression has been reported in rat ovaries (Sancho-Tello, *et al.*, 1992) and in mouse oocytes and follicular cells, in which the level of TNFα expression coincided with distinct steps of follicular development (Chen *et al.*, 1993). The initial phase of TNFα gene

transcription coincided with the formation of the second layer of granulosa cells. The source of TNFα appeared to be both macrophage-like cells and ovarian cells in both studies. Thus, TNFα may be involved in various stages of follicular development, atresia, and luteal function in the mouse (Terranova *et al.*, 1995). E. Sato *et al.* (1995) localised TNFα immunoreactivity in the cytoplasm of cumulus cells, surrounding mature oocytes, and suggested, based on the results of studies employing the addition of anti-TNFα antiserum and various concentrations of TNFα to oocytes *in vitro*, that TNFα might have an active role in preventing the degeneration of oocytes. In the hen, TNFα, in conjunction with a cholinergic agonist, was shown to promoted transmembrane Ca^{2+} fluxes resulting in increases in granulosa cell Ca^{2+} concentration (Soboloff *et al.*, 1995) that can potentially influence downstream differentiative signalling pathways that are important in oocyte maturation.

2.7.2. TNFα in the uterus

TNFα is expressed in the endometrium throughout the human menstrual cycle (Hunt *et al.*, 1992). TNFα mRNA was shown, in this study, to increase during the proliferative phase of the cycle, to decline during the early secretory phase, and to increase again during the mid to late secretory phases. It was later discovered, using a mouse model, that the sex hormones oestradiol and progesterone, have an influence on the cell-specific expression of TNFα and protein in the endometrium (Roby and Hunt, 1994) and myometrium (Roby and Hunt, 1995). Some potential roles for TNFα in the uterus were suggested, by these authors, including effects on the cyclic oedema that accompanies pro-oestrus and oestrus in the cycling uterus, leukocyte infiltration following oestrus, and apoptosis. Indeed, it has been demonstrated, recently, that TNFα expression in the endometrial epithelium increases up to, and including, the menstrual phase of the cycle and TNFR1, TNFR2, and Fas are also expressed (Tabibzadeh *et al.*, 1995). In addition, it was demonstrated, in the same study, that bcl-2 protein (an inhibitor of cell death) is also expressed. Thus, it was concluded that induction of apoptosis, by TNFα, and subsequent menstrual shedding may be related to the loss of the protective effect of bcl-2.

TNFα is synthesised by cells other than macrophages in both extraembryonic membranes and maternal tissue, including the uterus, during gestation (Yelavarthi *et al.*, 1991).

Furthermore, transcription of TNFα in specific types of cells is influenced by gestational age (Chen *et al.*, 1991). Roby and Hunt (1995) have suggested that, since TNFα can induce nitric oxide (NO) synthesis and NO helps to maintain the uterus in a relaxed state during pregnancy, TNFα could have a role in this process.

Recently, TNFα has been localised to activated natural killer (NK) cells in the pregnant mouse uterus (Parr *et al.*, 1995) and it has been suggested that TNFα, produced by NK cells, could have several roles in the pregnant uterus, including protection of the mother against aberrant foetal cells, restriction of trophoblast invasion of the maternal decidua, facilitation of the tissue remodelling required for accommodation of the embryo, and protection against the transmission of micro-organisms from the mother to the foetus. Furthermore, TNFα has been detected in horse trophoblasts and endometrium and it was suggested that TNFα might have a role in regulating growth and differentiation in the endometrium and placenta (Grünig and Antczak, 1995).

Alterations in uterine and amniotic TNFα levels may also have pathophysiological effects during pregnancy. Uterine cells, from diabetic rats, release significantly more biologically active TNFα than control cells and this is due to increased TNFα synthesis in the uterine epithelium (Pampfer *et al.*, 1995a). It has been demonstrated that the development of mouse embryos is inhibited *in vitro* by the products of activated macrophages (Hill *et al.*, 1987) and it was noted by Pampfer *et al.* (1995b) that the growth of day 5 rat embryos was significantly inhibited by culture medium conditioned with diabetic rat uterine cells and this effect could be reversed by antibodies to TNFα. However, it was not clear in this study whether dysregulated uterine TNFα production contributed towards, or was a consequence of, diabetes in these animals. It was concluded that excessive uterine production of TNFα during diabetes may contribute to the developmental deficiencies seen in preimplantation embryos from diabetic rats. Also, during intrauterine diseases such as chorioamnionitis, levels of cytokines, including TNFα, are altered and this can result in intrauterine growth retardation and perinatal death (Stallmach *et al.*, 1995).

2.7.3. TNFα in the placenta and during parturition

Jäättelä *et al.* (1988) first detected TNFα in amniotic fluids and in the supernatants of decidual and placental tissues, and demonstrated that TNFα levels were higher in the placenta during the second trimester than the third trimester of pregnancy. However, this placental TNFα was in a biologically inactive form, whereas the placental and decidual supernatants contained biologically active TNFα. Subsequently, Opsjon *et al.* (1993) showed that levels of IL-1, IL-6, and TNFα increase with the onset of labour and it was hypothesised that a low-level inflammatory response may occur in the term placenta, which may result in the production of cytokines, including TNFα, that regulate parturition. The inflammatory response could be caused by exposure of the foetal membranes to microbes from the vagina as the cervix dilates towards term. The induction of TNFα gene expression, at term, results in increased PGE_2 synthesis, which initiates parturition, and the tendency for TNFα to promote the onset of labour is significantly inhibited by TGFß (Bry and Hallman, 1992). Localised inflammatory responses may also lead to microscopic disruption of the amnion, which contributes towards preterm delivery, and this can be diagnosed by the presence of TNFα in the lower genital tract (Inglis *et al.*, 1994).

Membrane bound and sTNFRs have also been detected in human placentas (Austgulen *et al.*, 1992; Yelevarthi and Hunt, 1993). Gestation-related cell-specific differences in the levels of TNFR1 and TNFR2 mRNA and protein were noted. TNFR1 expression was predominant throughout gestation, both in mesenchymal and trophoblast cell lineages, while TNFR2 mRNA was present only in first trimester syncitiotrophoblasts and term placental mesenchymal cells. In addition, the expression of both TNFRs increased at term. Since, both soluble and membrane bound TNFRs were detected in these studies, it was suggested that placental TNFRs could have functions both in modulating the effects of TNFα in certain placental lineages during pregnancy (membrane-bound TNFRs) and in providing protection against excessive TNFα production during infections (sTNFRs).

2.7.4. TNFα and TNF receptor expression in embryos

The presence of inflammatory cytokines, including TNFα, in developing embryos is only just beginning to be appreciated and the functions of most of them are essentially unknown.

A range of inflammatory cytokines has been detected at early stages of development. Rothstein *et al.* (1992) have constructed cDNA libraries from various stages of pre-implantation mouse embryos and have detected transcripts of IL-6, IL-1β and IFN-γ. These workers point out that these cytokines not only regulate proliferation, but also induce differentiation. This is also true of TNFα, which is a member of the system of cytokines that includes the above-mentioned factors. Indeed, Kohchi *et al.* (1991) have detected the expression of TNFα and TNFß transcripts in murine embryonic carcinoma cells, which provide a model system analogous to pre-implantation embryos. This group has shown that TNFα is secreted during foetal and neonatal development of the mouse and they have proposed a model for the role of TNFα in development, known as "ontogenic inflammation", which is based on the cytokine network, present during inflammation (Yamasu *et al.*, 1989; Kohchi *et al.* 1994). These observations could be indicative of the presence, during development, of an array of inflammatory cytokines, such as: ILs; IFNs; and GM-CSF, all of which have known roles in inflammation. Kohchi *et al.* (1991) investigated this possibility, and detected the expression of GM-CSF, IL-1ß, IL-3 and IFN-γ in murine embryonal carcinoma cell lines, and a trophoblast cell line, thus supporting such a suggestion. Furthermore, Ohsawa and Natori (1989) observed the expression of a 25kDa form of TNFα in mouse embryos, which could be related to the 26kDa membrane bound form (Kriegler *et al.*, 1988), and speculated on a role for TNFα in programmed cell death (see Chapter 3). Gendron *et al.* (1991) reported upon the expression of high molecular weight proteins that are cross-reactive with TNFα antibodies in the developing nervous systems of mouse and chick embryos and speculated that these proteins may have a role in differentiation and proliferation. This is possible, since a range of inflammatory cytokines has been identified in nervous tissue in both pathological and physiological states (see above). Hunt *et al.* (1993) have identified TNFα mRNA and protein in mouse embryos and Jaskoll *et al.* (1994) have demonstrated that TNFα at 17kDa, as well as

a range of higher molecular weight TNFα-related proteins and TNFRs, are expressed in the developing mouse embryo lung.

Speculation on a role for cytokines in development has often been based on studies of the interaction of the immune and reproductive systems during pregnancy (see above). TNFα was found in the conditioned medium of 6-8 cell stage mouse embryos and it has been hypothesised that TNFα may have an effect on the endometrial inflammatory response, which is conducive to embryo implantation and placental development (Zolti *et al.*, 1991). TNFα is present in embryo culture fluids during *in vitro* fertilisation (Witkin *et al.*, 1991), suggesting that TNFα is secreted by embryos. It was suggested by these authors that TNFα secreted by embryos could have a role as a growth factor, it may regulate gene expression in the uterus, and it may aid embryo survival by inhibiting NK cell-mediated cytotoxicity and by inhibiting the NK-cell response to foetal antigens. A study by Lachapelle *et al.* (1993) reveals that preimplantation mammalian embryos secrete TNFα and may be considered to be TNFα resistant cell lines, up to the blastocyst stage, because they appear to lack TNFRs. This not only suggests a mechanism whereby the embryo can evade the host's immune system, but also implies that, at particular stages, embryonic cells may be resistant to their own developmentally regulated production of TNFα. Upregulation of TNFRs at a later stage of development could then make them sensitive to the pleiotropic effects of TNFα and Pampfer *et al.* (1994) showed that this is indeed the case, since mouse blastocysts were shown to express TNFR1. Therefore, it appears that TNFR expression is up-regulated at the morula-blastula transition stage. Interestingly, this also suggests that the response of embryonic cells to TNFα (cytotoxic or proliferative), may also depend on the specific TNFR up-regulated (see above). Indeed, Pampfer *et al.* (1994) found no evidence for increased cell death due to TNFα in the inner cell mass of the blastocyst, but these authors did observe a reduction in blastocyst cell proliferation mediated by TNFR1, which could be abrogated using an antisense oligonucleotide to TNFR1 (Pampfer *et al.*, 1995). In addition, sTNFRs have recently been identified in embryo culture fluids (Austgulen *et al.*, 1995). Thus, the potential for embryos to modulate TNFR and sTNFR expression could have far reaching implications for the different responses of embryonic cells to TNFα.

2.8. Avian TNFα-like factors

Since the model system for the studies described in this thesis is the chick embryo, it is important to review what is known about avian-TNFα like factors. Despite the fact that, in addition to mouse and human genes, rat, canine, feline, and porcine TNFα genes have been cloned and sequenced (Kwon *et al.*, 1993; Zucker *et al.*, 1994; Rimstad *et al.*, 1995; Pauli, 1995), there is, as yet, no information on the sequence of avian TNFα, because attempts to clone the gene in chicken systems have not been successful (Qureshi *et al.*, 1993).

However, avian equivalents of a number of mammalian cytokines have been recognised, including chicken TNFα-like factors (Klasing, 1994). Gendron *et al.* (1991) first identified TNFα immunoreactive proteins of 50kDa in the developing chick embryo nervous system using anti-mouse TNFα. Qureshi *et al.* (1993) and Zhang *et al.* (1995) have subsequently identified an avian TNFα-like factor, produced by chicken macrophages, in response to LPS, that induces cytotoxicity in cells of chicken lineage, but which fails to do so against mammalian targets, suggesting that this factor may be species specific. In addition, Byrnes *et al.* (1993) have identified a chicken TNFα-like factor produced in response to parasitic infection.

2.9. Concluding comments and statement of the thesis

An overview of the recent literature, pertaining to TNFα-mediated effects in physiology and pathophysiology, has been provided. This review has emphasised the plethora of processes that TNFα and its receptors, and their related molecules, are involved in and has highlighted the complex signalling pathways, initiated by TNFR binding by TNFα, and the role of other cytokines in the regulation of TNFα-mediated effects.

In addition, TNFα has been implicated in the development of the thymus, in haematopoiesis, and it is expressed in embryos at various stages. Studies in which TNFRs or TNFα have been " knocked out", however, suggest that TNF α may be dispensable during development. On the other hand, because of its highly pleiotropic nature, it is difficult not to envisage roles for TNFα in some aspects of embryonic development and the thesis put forward here is that TNFα has an important role in early embryogenesis in the chicken.

Furthermore, despite the studies revealing the presence of TNFα, and other inflammatory cytokines in developing embryos, virtually nothing is known of their detailed spatio-temporal expression patterns during embryonic development and the specific functions, they may have in the cells and tissues in which they are expressed. Therefore, the main objective of this thesis is to gain a greater understanding of in which tissues TNFα is expressed and its roles in these tissues during embryonic development. In this respect, the hypothesis put forward in Chapter 3 will be useful.

TABLE 2-1.

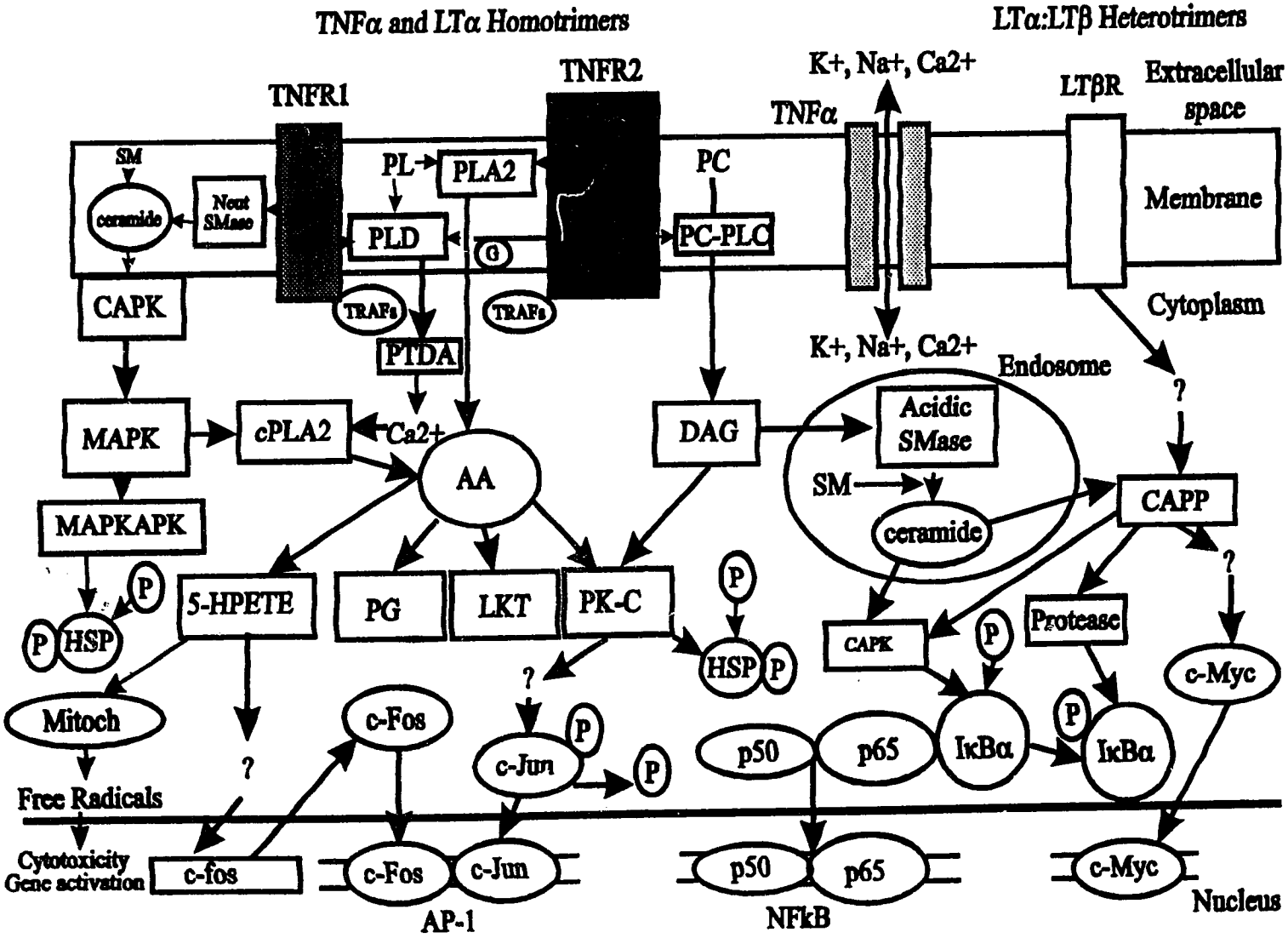
Summary of TNF ligand and receptor family members and the effects of TNF ligand or receptor knockouts or naturally occurring mutations.

(Modified from Warzocha *et al.*, 1995; Beutler, 1995).

RECEPTOR	*LIGAND*	*CELL RESPONSE*	*PHYSIOL-OGICAL ROLE*	*LIGAND KO OR MUTATION*	*RECEPTOR KO OR MUTATION*
TNFR1 (55kDa)	TNFα, LTα, $LT\alpha_2:LT_2\beta_1$	apoptosis, proliferation, differentiation	1. T- and B-cell activation 2. T- and B-cell differentiation		1. Reduced immunity to intracellular pathogens. 2. Reduced ability to respond to LPS
TNFR2 (75kDa)	TNFα, LTα, $LT\alpha_2:LT\beta_1$	apoptosis, proliferation, differentiation	1. T- and B-cell activation 2. T- and B-cell differentiation		Reduced dermal responses to TNFα
LTβ-R	$LT\alpha_2:LT\beta_1$	?	Formation of peripheral lymphoid organs	Absent lymph nodes and disordered splenic architecture	NDA
Fas (Apo)	FasL	apoptosis	1. Immune tolerance 2. T-cell mediated cytotoxicity	1.Lymphade-nopathy. 2. Increased numbers of CD3+, CD4-, and CD8- T cells. 3. Autoimmunity	As ligand mutation
CD27	CD70	differentiation, proliferation	Antigen driven T-cell activation and proliferation	NDA	NDA
CD30	CD30L	apoptosis, proliferation, differentiation	1. T-cell proliferation. 2. B-cell maturation	NDA	NDA
CD40	CD40L	anti-apoptosis	T-cell dependent B-cell proliferation and Ig class switching	1. Increased plasma IgM. 2. Reduced IgG, IgA, IgE	NDA
4-1BB	4-1BBL	proliferation	Antigen-presenting cell driven T-cell activation and proliferation	NDA	NDA
OX-40	OX-40L	proliferation	T-cell activation	NDA	NDA
p75 NGFR	NGF	anti-apoptosis	Development of peripheral nerves	Deficits in peripheral sensory nerve development	

NDA = no data available

Figure 2-1. TNFα-Induced Signalling Pathways.
(After Heller and Kronke, 1991; Warzocha *et al.*, 1995)



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CHAPTER 3

HYPOTHESIS: POTENTIAL ROLES FOR TNFα DURING EMBRYONIC DEVELOPMENT[1]

TNFα has well known roles in pathophysiology (Vassalli, 1992; see Chapter 2), but very little is known about its potential roles in physiology, although it is expressed at high levels in the tissues of normal individuals (Tovey *et al.*, 1988). It was suggested that a possible reason for the conservation of the TNFα gene through evolution (Ahne, 1994) is that, in addition to its beneficial roles in immunity and infection (see Chapter 2), it has an important role in development (Beutler and Cerami, 1986; Tracey and Cerami 1990).

In this Chapter, some of the potential roles for TNFα in development, based on its known effects in inflammation and immunity, are considered. These speculations provide the basis for the studies that comprise the remaining Chapters of this thesis and which contribute to a greater understanding of the role of TNFα-like proteins in embryonic development.

3.1. A role in programmed cell death

3.1.1. Historical background

The naturally occurring death of cells is a phenomenon that has a long history (Clarke and Clarke, 1995), even though it is only relatively recently that it has risen to the top of the biological research agenda. The first account of cells dying during development was provided by Vogt (1842) who described the death of cartilaginous and notochordal cells. Other landmark discoveries were those of Beard (1896) who described cell death in the nervous system during metamorphosis, and Felix (1889) who first described scattered cell death in developing mammalian muscle (the first description of cell death in a system destined to remain). Glücksmann (1951) provided the first comprehensive review of naturally occurring cell death. This was a very important piece of work, because it made the literature accessible

[1] *A version of this Chapter has been published as part of Wride M A and Sanders E J. (1995) Potential roles for tumour necrosis factorα during embryonic development. Anat. Embryol. 191: 1-10.*

to the English speaking world, since the majority of the early work was published in German. The other review of historical interest is that of Saunders (1966) who reviewed the control mechanisms for cell death as they were understood at the time. As Sanders and Wride (1995) have pointed out, this proved to be a very insightful piece of work in the light of the developments which have occurred over the last thirty years in the understanding of the regulation and control of cell death phenomena.

3.1.2. Programmed cell death and apoptosis

The term 'programmed cell death" was introduced by Lockshin in a series of papers concerned with the degeneration of intersegmental muscles during silkmoth metamorphosis (Sanders and Wride 1995). Programmed cell death is a term used mainly, but not exclusively, by developmental biologists and implies that there is a genetic programme that is intrinsic to an organism during its development which results in the death of cells at specific times and in specific locations. The classic example of this type of cell death is that occurring during the development of the nematode worm *Caenorhabditis elegans* (Sanders and Wride, 1995).

Apoptosis was a term introduced to the literature by Kerr *et al.* (1972) to describe the appearance of cells dying during experimentally-induced hepatic ischemia, which were distinct from cells dying by 'necrosis" or non-physiological cell death, in the same experiment. The term apoptosis was coined to imply that cell death, by this mechanism, was the opposite of mitosis in terms of the regulation of tissue size. Like mitosis, the word is derived from Greek and means 'falling off', i.e. as leaves from a tree (Kerr and Harmon, 1991). These authors also point out that a similar process occurs in plants, implying that apoptosis originated very early in evolution. Apoptosis has a characteristic set of morphological features that distinguish it from other types of cell death, such as necrosis, and other types of physiological cell death, such as those occurring during metamorphosis (Sanders and Wride, 1995). These features include: condensation of the chromatin, fragmentation of the nucleus, vacuolation, swelling of endoplasmic reticulum cisternae, rupture of mitochondria, and exocytosis of fluid-filled vacuoles (Kerr and Harmon, 1991; Sanders and Wride, 1995).

Although apoptosis also characterises cell death in a number of pathological and immunological situations, it is a type of cell death that often, though not exclusively,

characterises the programmed cell death observed during embryonic development[2]. At first, it seems counter-intuitive to postulate a role for cell death in a constructive process like development. However, although there is argument at the present time about the molecular details of the process; i.e. if all types of programmed cell death are truly genetically "programmed", as they appear to be in invertebrates such as *Caenorhabditis elegans* (Driscoll and Chalfie, 1992; Sanders and Wride, 1995), and if all types of programmed cell death are accompanied by DNA fragmentation (Tomei *et al.*, 1993), it now receives the wide recognition it has long deserved as a legitimate morphogenetic concept with an essential role in the development of many tissues in many species (Hinchliffe, 1981; Snow, 1987; Sanders and Wride, 1995). However, the identity of many of the proteins involved in cell death during development remains unknown.

3.1.3. TNFα and apoptosis

TNFα has the ability to induce apoptosis in both transformed and normal cells (Laster *et al.*, 1988; Robaye *et al.*, 1991; Wright *et al.*, 1992) either on its own or through synergistic action with factors such as IFN-γ (Jo *et al.*, 1995; Kalovidouris and Plotkin, 1995) Conversely, factors such as TGFβ may protect against TNFα-mediated apoptosis (Chang *et al.*, 1995).

Following binding to its receptors, TNFα sets in motion many intracellular pathways (see Chapter 2). Those involved in cytotoxicity include phospholipase activation, generation of free radicals, activation of endogenous endonucleases (Larrick and Wright, 1990; Beyaert and Fiers, 1994) mobilisation of Ca^{2+} (Belloma *et al.*, 1992), activation of multiple proteases (Higuchi *et al.*, 1995; Voelkel-Johnson *et al.*, 1995), and modulation of topoisomerase activities (Baloch *et al.*, 1995).

Apoptosis has an important role in immunity (reviewed by Cohen and Duke, 1992) and it is becoming apparent that various forms of TNFα are involved in apoptosis. Degliantoni *et al.* (1985) showed that natural killer cell (NKC) cytotoxic factor is related to TNFα produced

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[2] *A detailed discussion of whether the terms "apoptosis" and "programmed cell death" are interchangeable will not be embarked upon here. The reader is referred to the review of Sanders and Wride (1995) for a more detailed discussion on this point and for examples of programmed cell death that do not have the classic apoptotic features.*

by monocytes or myeloid cell lines. More recently, it has been shown that various high molecular weight membrane-bound forms of TNFα are involved in the cytotoxicity mediated by both NKCs and cytotoxic T-lymphocytes (CTLs). Liu *et al.* (1989) identified TNFα-related cytokines at 50-60kDa, which they called "leukalexins", in the plasma membranes of CTLs, while Khinkhabwala *et al.* (1990) also identified high molecular weight forms of TNFα on the surfaces of activated T-cells. Furthermore, recent reports have identified and characterised a number of higher molecular weight molecules that belong to the family of TNFα-related proteins (see Chapter 2). Several of these ligands can induce cell death following interaction with their receptors; for example, Fas and FasL (Bazan, 1993; and see Chapter 2), and a number of them are approximately 50kDa in size, which may be relevant to the findings described in Chapter 4.

Most recently, it has been revealed that the product of *reaper*, a gene involved in programmed cell death in the *Drosophila* embryo (White *et al.*, 1994; White and Steller, 1995), is homologous to the cell death-promoting domains found in Fas and TNFR1 (Golstein *et al.*, 1995; Hofmann and Tschopp, 1995; see Chapter 2). Thus, the reaper cell death domain, present in Fas and TNFR1, may have been conserved during evolution because it has an important role in programmed cell death during development.

Therefore, considering the fact that TNFα has the ability to cause apoptosis in sensitive cells, that TNFα and its receptors have been identified in embryos and that programmed cell death (frequently characterised morphologically as apoptosis) occurs extensively during embryonic development, it is suggested here that proteins with TNFα-like activity could be cell death promoting proteins expressed during developmentally programmed cell death.

3.2. A role as a growth and differentiation factor

TNFα can behave as a growth factor (reviewed by Vilcek and Palombella, 1992) and as a proliferation factor (Sugarman *et al.*, 1985) and it can induce a number of other cytokines, growth factors and their receptors, including: activin A in bone marrow (Shao *et al.*, 1992; Takahashi *et al.*, 1992), leukaemia inhibitory factor (LIF) by human articular chondrocytes and cartilage (Campbell *et al.*, 1993), nerve growth factor (NGF) in non-neuronal cells (Hattori *et*

al., 1993), the epidermal growth factor receptor (EGF-R) on glioma cells (Adachi *et al.*, 1992) and transforming growth factor α (TGFα) in pancreatic cancer cells (Schmiegel *et al.*, 1993). These factors all have known roles in development. Activin is involved in the induction and patterning of mesoderm (reviewed by Stern, 1992) and in the chondrogenesis of chick limb bud mesodermal cells (Chen *et al.*, 1993), while LIF has been shown to induce nephrogenesis in the mouse kidney (Bard and Ross, 1991), to inhibit mouse gastrulation (Conquet *et al.*, 1992), and is involved in the development of nervous tissue (Richards *et al.*, 1992; Murphy *et al.*, 1993). NGF acts as a trophic factor in the nervous system (Shigeno *et al.*, 1991; Mesner *et al.*, 1992) and the p75 NGFR, which belongs to the same family that includes the TNFRs and the related Fas/Apo-1 antigen (Oehm *et al.*, 1992; see Chapter 2), is expressed during kidney morphogenesis (Sariola *et al.*, 1991). EGF and TGFα are required for kidney tubulogenesis *in vitro* (Taub *et al.*, 1990) and TGFα and the EGF-R have been localised immunocytochemically in developing chicken kidneys (Díaz-Ruiz *et al.*, 1993), suggesting a role for them in proliferation and differentiation in this tissue. The fact that TNFα is capable of influencing the expression of these factors in various situations could imply an involvement of TNFα in their regulation during development. Furthermore, TNFα has been implicated directly in the proliferation of mouse primordial germ cells in culture, suggesting a possible involvement of TNFα in the proliferative regulation of primordial germ cells in the embryo (Kawase *et al.* 1994).

3.3. A role in remodelling of extracellular matrix and induction of cell adhesion molecules and integrins

TNFα has a pivotal role in the inflammatory response, particularly in its ability to affect the synthesis and degradation of ECM molecules. Furthermore, it has been proposed that TNFα may also have a physiological role in the co-ordinated removal and replacement of senescent ECM components during normal tissue homeostasis (Vlassara *et al.* 1988). It is therefore possible that TNFα may also serve similar functions during embryonic development, since this process is characterised by high rates of remodelling of ECM.

TNFα has been shown to induce several proteins, which have known roles in development, in various situations. TNFα induces fibronectin synthesis by fat storing cells in the rat liver during fibrinolysis (Bachem *et al.*, 1993), glycosaminoglycans in cultured vascular smooth-muscle cells (Kaji *et al.*, 1993), hyaluronan synthesis in lung fibroblasts (Sampson *et al.*, 1992), and it has a modulatory effect on proteoglycans (reviewed by Nietfield, 1993). Furthermore, TNFα can mediate an increase in endothelial cell permeability by decreasing cell-ECM contacts, which may result in the elongation of cells and in the formation of intercellular gaps (Partridge *et al.*, 1992), and it can induce the production of various proteinases in inflammatory joint disease (Smith *et al.*, 1992; Huet *et al.*, 1993). In addition, TNFα enhances stromelysin-1 and collagenase expression in dermal fibroblasts and suppresses type 1 collagen mRNA levels (Westermarck *et al.*, 1995).

The protease, urokinase has been implicated in the epithelial-mesenchymal transition of the somites (McGuire and Alexander, 1992) where it is suggested to have a role in cell migration and matrix-remodelling during development of the axial skeleton. In this connection, Niedbala & Stein-Picarella (1992) have shown that TNFα mediates an increase in urokinase mRNA, and that increased cell surface urokinase expression correlated with an increased ability of endothelial cells to invade matrix. This is an event perhaps analogous to the invasion of the basement membrane by the future mesoderm cells of the epiblast at the primitive streak during gastrulation and sclerotome invasion of the perinotochordal matrix.

TNFα also down-regulates the $\alpha_6\beta_1$ integrin (laminin receptor) in endothelial cells during inflammation (Defillipi *et al.*, 1992) and induces a wide range of CAMs in various inflammatory situations (reviewed by Mackay and Imhof, 1993). CAMs are usually expressed during inflammation in order to recruit inflammatory cells and platelets to injured sites (Arnaout, 1993). Vascular CAM-1 (VCAM-1) is a member of the immunoglobulin family and is involved in immune cell maturation and function (Rosen *et al.* 1992), while intercellular adhesion molecule-1 (ICAM-1) is a glycoprotein that is also involved in interactions between cells of the immune system and in inflammation (Wertheimer *et al.* 1992; Couffinhal *et al.* 1993). TNFα induces VCAM-1 on endothelial cells (Briscoe *et al.*, 1992), in fibroblast-like cells from synovial tissue (Marlor *et al.*, 1992), and in renal tubular epithelial cells (Wuthrich *et*

al., 1993). It also induces ICAM-1 in vascular smooth muscle cells (Couffinhal *et al.*, 1993) and in endothelial cells (Myers *et al.*, 1992; Wertheimer *et al.*, 1992), and induces the mouse endothelial E- and P-selectins (Weller *et al.*, 1992), and a range of CAMs in foetal astrocytes (Hurwitz *et al.*, 1992). CAMs have an essential role in cell interactions during development (reviewed by Edelman and Crossin, 1991; Takeichi, 1988). Since VCAM-1 is expressed on differentiating skeletal muscle (Rosen *et al.*, 1992) and TNFα immunoreactivity is present in the developing chick myotome (see Chapter 4), it is possible that TNFα could promote VCAM-1 expression at certain stages of myogenesis. However, Miller *et al.* (1988) have shown that TNFα decreases human myogenesis *in vitro* during a narrow temporal window of differentiation, suggesting possible stage-specific effects of TNFα on myogenesis, which may or may not occur through CAMs.

3.4. Other potential roles

3.4.1. Influence of TNFα on ubiquitin

Ahrens *et al.* (1990) have shown that TNFα can enhance the induction, by ß-interferon, of a ubiquitin-cross-reactive protein in the development of the anti-viral response in some cell lines, while García-Martinez *et al.* (1993) have shown that TNFα increases the ubiquitinisation of rat skeletal muscle proteins. Ubiquitin is a highly conserved protein with several roles in eucaryotic cells, the most well known being its role in proteolysis (Hochstrasser, 1992). It also has a role in the programmed cell death of muscle during insect metamorphosis (Sanders and Wride, 1995) and is present in the embryonic chicken nervous system at the time of motoneuron degeneration (Schwartz, 1991). Scotting *et al.* (1991) and Smith-Thomas *et al.* (1994) have investigated immunohistochemically the distribution of ubiquitin-protein conjugates in the developing chick embryo. These proteins were detected in the lens, nervous system, notochord and myotome at similar stages to the detection of TNFα immunoreactivity in these tissues (see Chapter 4). Therefore, there could be a link between the expression of TNFα-like molecules and the expression of ubiquitin. In this scheme TNFα, along with other cytokines, such as IFNs, could initiate the expression of proteins involved in the rapid removal of cellular proteins during programmed cell death, and cell differentiation,

during development of tissues such as the myotome, the notochord, the lens, and the nervous system.

3.4.2. Protection of embryonic cells from TNFα-mediated cytotoxicity and a role for TNFα in a "free radical theory of development"

Induction of the expression of genes conferring resistance to TNFα cytotoxicity may provide a means of regulating the differential effects of TNFα on particular cells during development; for example, a differentiative or proliferative response instead of an apoptotic response. TNFα can increase the formation of toxic free radicals, and it can also induce synthesis of proteins, such as manganous superoxide dismutase (MnSOD), metallothionein and ferritin (Wong *et al.*, 1992), which protect against toxicity due to oxygen metabolites (see Chapter 2). Indeed, it has been shown that endogenous TNFα can protect cells against the cytotoxic effects of exogenous TNFα by inducing MnSOD (Okamota *et al.* 1992). This protection may not be solely restricted to proteins, but it may also extend to gangliosides, which have been shown to be protective against the apoptotic effects of TNFα by perhaps interfering with TNFα-mediated signal transduction (Koike *et al.*, 1993). It is interesting, in the light of this, that gangliosides are similar in structure to LPS, a potent stimulator of TNFα production, and may be involved in the induction of TNFα and components of the complement system during what has been termed "ontogenic inflammation" (Kohchi *et al.*, 1992; see Chapter 2).

Reactive intermediates of oxygen metabolism may affect developmental events by setting up metabolic, redox, and ionic gradients in embryos, which influence gene expression and the control of developmental processes (reviewed by Allen & Balin, 1989). In view of this and the ability of TNFα to induce free radicals, it is also possible that TNFα could regulate free radical production and effect in embryos, thereby influencing various developmental processes. For example, the study of Schulze-Osthoff *et al.*, (1993) reveals that reactive oxygen intermediates produced by mitochondria, in response to TNFα, are not only involved in cytotoxicity, but can also act as second messengers in inducing activation of the important transcriptional activator NFκB (see Chapter 2).

3.4.3. Influence of TNFα on the cytoskeleton

It has been demonstrated that TNFα can affect actin stability in a number of cell types. For example, TNFα promotes F-actin depolymerisation and re-organisation in endothelial cells (Goldblum *et al.*, 1993; Deli *et al.*, 1995), resulting in morphological changes that bring about increases in the extent of intercellular spaces and endothelial permeability. In addition, β-actin mRNA has been shown to be de-stabilised by TNFα in human microvascular endothelial cells (Kohno *et al.*, 1993). In endometrial epithelial cells, TNFα induced disassembly of actin filaments in a dose-dependent manner resulting in cell-cell dissociation (dyscohesion) through effects on Rho and cadherins/β-catenin (Tabibzadeh *et al.*, 1995a,b).

The influence of TNFα on actin stability has a number of implications for events occurring during embryogenesis, including cell migration, cell elongation, and a type of apoptotic cell death termed "anoikis", which can occur as a result of alterations in cell adhesiveness (Frisch and Hunter, 1994). Indeed, recent studies looking at cells from the gastrulating chick embryo in culture (Sanders *et al.*, 1996) show that both TNFα and TNFR2 are associated with actin stress fibres and that TNFα promotes cell-matrix adhesion and protects against "anoikis", since the addition of anti-TNF α increases the incidence of cell death, as assessed by TUNEL, a method for detecting cells undergoing the DNA fragmentation associated with cell death (see Chapter 5).

3.5. Concluding comments

It is proposed here that TNFα may have three major roles during embryonic development: in programmed cell death; in the regulation of cellular growth and differentiation; and in the remodelling of ECM and the regulation of CAMs and integrins. These three roles are analogous to those that TNFα has in tissue remodelling in inflammation and to its role both as a mediator of apoptosis and as a growth and differentiation factor in the immune system.

In conclusion, it is clear from the literature reviewed in Chapter 2 that TNFα is expressed in embryos in various tissues and at various stages. It remains now to elucidate the functions and importance of this highly pleiotropic molecule during embryonic development, and to determine how it might interact with the network of other cytokines and growth factors

that are also present during embryogenesis. In order to achieve this aim, the hypothesis put forward here may be useful. With specific regard to this thesis, this hypothesis is used as the basis for the approach used in subsequent Chapters.

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PART II: IMMUNOLOCALISATION AND IMMUNOREACTIVITY

CHAPTER 4

EXPRESSION OF TNFα-CROSS-REACTIVE PROTEINS DURING EARLY CHICK EMBRYO DEVELOPMENT[1]

4.1. Introduction

TNFα is produced primarily by monocytes and macrophages and it plays a pivotal role in the physiological and pathological consequences of inflammation, infection and invasion by altering host metabolism (Vassalli, 1992). Along with other cytokines, such as IL-1, it is a major mediator of fever and is produced in auto-immune disorders, and in a wide range of inflammatory diseases (Jacob, 1992). The net biological effects of TNFα vary according to its tissue levels. At high concentrations it is detrimental to the host: in toxic shock, and cancer cachexia; whereas at low concentrations its effects are beneficial: in tissue remodelling, inflammation and host defence (Tracey, 1992).

In addition to its roles in inflammation and cellular immune responses, TNF has

regulate the expression of $\alpha_6\beta_1$ integrin (laminin receptor; Defilippi *et al.*, 1992) in human endothelial cells, and it can facilitate the remodelling of the ECM (Partridge *et al.*, 1992).

It has also been established that TNFα has the ability to cause apoptosis in transformed cells (Laster et al., 1988; Wright *et al.*, 1992), and normal cells (Robaye *et al.*, 1991). Apoptosis is a type of cell death identified morphologically by early nuclear compaction and cytoplasmic condensation in membrane bound apoptotic bodies. This is often followed by fragmentation of the nucleus. Subsequently, the cell remnants may be removed by phagocytosis or digested by unaffected adjacent cells (Kerr *et al.*, 1987; Gerschenson & Rotello, 1992).

Apoptosis is characteristic of the programmed deletion of cells observed during development (Hurle, 1988), and it is widely recognised that cell death has a vital role in the differentiation of many tissues and organs in a wide variety of species (Snow, 1987; Ellis *et al.*, 1991; Sanders and Wride, 1995). Several developing systems are particularly noted for the importance of cell death, these include: the nervous system (Oppenheim, 1991; Hamburger, 1992), and the limb buds, which have provided a particularly useful system in which to study this phenomenon (Hinchliffe, 1981).

The beneficial effects of the TNFα gene must outweigh the detrimental effects, since it has been conserved through evolution. To explain this, Vlassara *et al* (1988) suggested that the ability of TNFα to regulate cell death, growth and proliferation indicates a role for it in normal tissue remodelling in the adult. With this in mind, and the fact that programmed cell death is of considerable developmental significance, another possible reason for the conservation of the TNFα gene, despite the risks associated with its presence in the genome, is that it has a role in embryonic development (Beutler & Cerami, 1986; Tracey & Cerami, 1990).

There is already some evidence implying a role for TNFα-like proteins in development. Ohsawa & Natori (1989) searched for TNFα transcripts in mouse embryos, using Northern hybridisation, and detected transient expression of TNFα mRNA in extracts of embryos on days 9 and 10 of gestation. They also identified a 25kDa protein, showing TNFα cross-reactivity, using immunoblotting analysis. This is greater than the size of monomeric TNFα from macrophages (17kDa; Sprang & Eck, 1992), and they suggested that this form of the

molecule could be an integral membrane protein (Kriegler, *et. al.*, 1988; Luettig, *et. al.*, 1989) with biological activity, since no 17kDa band was detected. Ohsawa and Natori (1989) speculated that TNFα could have a role in the initiation of programmed cell death during development. Gendron *et al.* (1991), have immunocytochemically localised TNFα-cross-reactive proteins in nervous tissue of mouse (days 10-15), and chick embryos (days 8-16). In the former, TNFα cross reactivity was detected in the brain, neural tube, and peripheral mixed spinal nerves, while in the avian embryo TNFα was detected in the brain neuroepithelium and Purkinje neurons of the cerebellum. Once again, a molecule larger than 17kDa was detected in Western blots of both species, however, it was larger (50kDa) than that identified by Ohsawa & Natori. It was concluded that expression of this protein could be involved in the development of specific components of the central and peripheral nervous systems. Also, Clough *et. al.*, (1992) have detected transcripts of several cytokines, as well as TNFα immunoreactivity, during development of the mouse brain and, more recently, Jaskoll *et al.* (1994) have identified TNFα and its receptors in the developing mouse lung and it was suggested that TNFα may have a role in lung branching morphogenesis.

In this Chapter, observations are presented that support a role for TNFα-cross-reactive proteins in embryonic development at earlier stages than those previously studied. Using a polyclonal and two monoclonal antibodies to mouse TNFα, immunocytochemistry has been carried out on the early chick embryo, from 1 to 6 days of embryonic development (stages 5 - 29 of Hamburger and Hamilton, 1951). Proteins showing TNFα-cross-reactivity are shown to have a widespread distribution at these stages, being expressed mainly in the mesoderm and endoderm during gastrulation and at later stages in the lens, notochord, sclerotome, myotome, neural tube, spinal and cranial nerves, as well as the mesenchyme of the head, trunk, and limb buds. The results suggest a role for TNFα-like molecules in the regulation of cell differentiation and perhaps cell death during early avian development.

4.2. Materials and methods

4.2.1. SDS-polyacrylamide-gel-electrophoresis

Embryos were removed from their yolk, and rinsed and handled in Pannet and Compton's saline. Whole embryos or dissected tissues were immediately homogenised in tris-buffered saline (TBS) at 4°C at a volume-to-volume ratio of 1 part tissue to 5 parts TBS (pH 7.5). Samples were then aliquoted and stored at -70°C until use. Samples containing 40μg of protein were taken and made up to 20μl with TBS followed by addition of 20μl of 2 x O'Farrels sample buffer, pH 6.8 (0.177M SDS, 0.14M TRIS, in glycerol to a final concentration of 22% v/v), containing 1M dithiothreitol (DTT; at a ratio of 9:1). Finally, samples were heated at 95°C for 5 mins.

In one experiment, 6 day (stage 29) embryos were homogenised in TBS containing fresh glycosidase F (Boehringer Mannheim) at 0, 10, 20, and 30 Units/ml and incubated for 18 hrs at 37°C.

In addition, some embryos were homogenised in a protease inhibitor buffer (pH 7.8; Jaskoll *et al.*, 1994), which had the following composition: HEPES, 20mM; Magnesium chloride, 10mM; EDTA (2mM); EGTA, 0.2mM; and protease inhibitors aprotinin, 15 μg/ml; leupeptin, 1μg/ml; pepstatin, 5μg/ml (Sigma). Phenylmethylsulphonyl fluoride (PMSF; Sigma) was added to the buffer 2.5 hrs before use to give a final concentration of 174μg/ml. The protocol for homogenisation, when using the protease inhibitor buffer, was exactly as described above.

Protein concentration for each embryo homogenate was determined by the Bio Rad protein assay method and samples containing 20μg of protein were loaded onto each lane of a 15% SDS polyacrylamide gel, and electrophoresed for 1hr at 200v. Protein standards (Bio Rad) and a positive control of 15ng of recombinant mouse TNFα (Genzyme) were run simultaneously.

4.2.2. Western immunoblot analysis

Proteins were transferred from the gel to a nitrocellulose membrane (Bio Rad) for 2hrs at 100v. The nitrocellulose membrane was cut into strips, and incubated for 2hrs in 3%

skimmed milk (sm) in TTBS (TBS with 0.5% Tween, pH 7.5 with 0.01% thimerosal), while shaking at room temperature. Strips were then incubated overnight, at room temperature while shaking, in polyclonal anti-TNFα antibody at a dilution of 1:500 in 3% sm in TTBS. Controls were incubated in non-immune rabbit serum at 1:500, or in antibody preabsorbed by mixing 10μl (1:500) with 0.5μg of recombinant mouse TNFα (Genzyme) in 5mls of 3% sm, for 3hrs at room temperature. This was followed by three washes in 3% sm in TTBS. Strips were then incubated with secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, Bio Rad) at a dilution of 1:3000 in 3% sm in TTBS, for a further 2hrs while shaking at room temperature. Finally, strips were washed three times in 3% sm in TTBS and then three times in TBS. Colour reaction solutions were prepared immediately before application: 4-chloro-naphthol (Bio Rad; 6 mgs in 2mls methanol), was added to 10mls of 0.18% v/v H_2O_2 in TBS, pH 7.5, at room temperature. The mixture was then applied directly to the nitrocellulose strips for 5-10 mins. The reaction was stopped by immersion in distilled de-ionised water, and the strips dried.

Two monoclonal antibodies to mouse TNFα were also used in Western blot analysis. Rat anti-mouse TNFα, clone MP6-XT22, was obtained from either Endogen Inc. or abV ImmuneResponse Inc., while clone MP8-XT22 was obtained from UBI Inc. Strips were incubated overnight with each monoclonal antibody at a concentration of 100μg/ml, or with antibody pre-absorbed by mixing 100μl of monoclonal antibody with 0.5 μg of recombinant TNFα in 1ml of 3% sm as described above. This was followed by incubation of the strips for 1 hr with biotinylated goat anti-rat IgG (mouse absorbed; Dimension Laboratories Inc.) at a dilution of 1:100 in 3% sm. This was followed by washing as described above. Finally, strips were incubated for 1 hr with extravidin-peroxidase (Sigma) at a dilution of 1:200 in 3% sm. Washing and colour development were carried out as above except that the colour development was allowed to occur overnight in distilled de-ionised water containing a few drops of colour-development reagent.

In later experiments, the colourimetric protocol for immunoreactive band detection was replaced by a more sensitive chemiluminescent method using a detection kit from Amersham,

Inc. Details for TNFα immunoreactive band detection using chemiluminescence are provided by Jaskoll *et al.* (1994).

4.2.3. Preparation of embryos for immunocytochemistry

White leghorn hens' eggs were incubated for 1 to 6 days as required, and staged according to the table of Hamburger and Hamilton (1951). The embryos were removed from their yolk and rinsed and handled in Tyrode's saline. Specimens were fixed with 4% paraformaldehyde for 4 to 6 hrs at 4°C (methacarn and Bouin's fixatives were also used, and gave similar results) washed in phosphate-buffered saline (PBS, pH 7.4), dehydrated through a graded series of ethanol, cleared in xylene and embedded in paraffin wax. Blocks were sectioned at 8 μm thickness.

4.2.4. Immunocytochemical Staining for TNFα

For staining, sections were deparaffinised in Hemo-De (Fisher Scientific) and rehydrated through a graded series of ethanol. Slides were then washed in TBS (pH 7.6) for 5 mins, and immunostained using the alkaline-phosphatase based AS/AP® Plus kit (Bio/Can Scientific Inc.). Slides were incubated in the blocking agent provided for 1hr at room temperature to prevent non-specific binding. Polyclonal rabbit anti-mouse TNFα (neutralising) antibody (Genzyme) was then used at a dilution of 1:1,000 or 1:750 in TBS plus 5% goat serum. Control slides were incubated with the same dilution of non-immune rabbit serum. Incubation with primary antibody was carried out overnight at 4°C in a sealed, humidified chamber. Sections were then washed in TBS, and incubated with a goat anti-rabbit biotinylated IgG, used undiluted as provided in the kit for 30 mins, at room temperature. This was followed by a further wash in TBS, and incubation in enzyme conjugate for 30 mins at room temperature. Slides were washed and incubated in the chromogenic substrate solution for 10-15 mins at room temperature. Endogenous alkaline phosphatase activity was inhibited by the addition of 1 part levamisole to 14 parts of the AS/AP® chromogenic substrate solution just before use. Slides were then washed in TBS, and mounted immediately in AS/AP® mounting medium. Slides were dried in a horizontal position, in an oven at 60°C for 30 mins.

Areas which were immunoreactive against the TNFα antibody were stained with an intense brown deposit. All experiments were repeated at least three times under the same conditions to ensure reliability.

In addition, both of the rat monoclonal antibodies to mouse TNFα were used for immunocytochemistry. These antibodies were used as above, at a dilution of 1:20, and detected with the AS/AP® system in which the second antibody was replaced by biotinylated rabbit anti-rat IgG (Dimension Laboratories, Inc.) diluted 1:50 in TBS plus blocking agent.

4.2.5. Ultrastructural immunogold cytochemistry[2]

Pre-embedding ultrastructural immunocytochemistry was carried out using stage 5 embryos from which the endoderm layer had been removed, allowing the reagents access to the mesoderm cells. Embryos were cleaned free of yolk, dissected in PBS, and incubated in 1% millipored bovine serum albumin (BSA) for 30 mins. All steps in the procedure were carried out at 4°C. Embryos were transferred to the polyclonal anti-TNFα antibody (diluted 1:50 in PBS plus BSA) for 1 hr, followed by washing with BSA for 30 mins. Controls were incubated in either normal rabbit serum or PBS alone. All samples were then treated with protein A-gold (E-Y Labs.) at a dilution of 1:10 in PBS plus BSA for 1 hr and then washed in three changes of PBS for 15 mins each. Specimens were fixed with 2.5% phosphate-buffered glutaraldehyde, pH 7.4 at 4°C, overnight. After washing in PBS, the embryos were post-fixed with 1% buffered osmium tetroxide for 1 hr at room temperature, then washed, dehydrated, embedded in Araldite and sectioned. Grids were examined, either stained or unstained, using a Philips 300 electron microscope. Results were quantified by counting the number of gold particles on randomly selected 10μm stretches of cell membrane.

4.3. Results

4.3.1. Antibody specificity

The specific immunoreactivity of the polyclonal antiserum for TNFα was demonstrated by Western blot analysis. The polyclonal antibody reacted specifically with recombinant mouse

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[2] *Ultrastructural immunogold cytochemistry was carried out by Sita Prasad in conjunction with Dr E J Sanders.*

TNFα, which is known to have a molecular weight of 17kDa (Fig. 4-1a; lane 2), while antibody preabsorbed with recombinant mouse TNFα failed to detect this 17kD band (Fig. 4-1a; lane 1). Similarly, non-immune rabbit serum at the same dilution gave a negative result (data not shown). The specific immunoreactivity of the monoclonal antibodies for TNFα was confirmed by pre-absorbing each of them with recombinant mouse TNFα (Fig. 4-3).

4.3.2. Western blot analysis

The expression of chick embryo proteins showing cross-reactivity with the polyclonal TNFα antibody was demonstrated by immunoblotting of whole-embryo homogenates as shown in Fig. 4-1a. The 17kDa monomeric form of TNFα was not detected at any stage examined when not using the protease inhibitor buffer; however, immunoreactive molecules of approximately 50kDa and 70kDa were identified. Antibody pre-absorbed with recombinant mouse TNFα failed to detect immunoreactivity in either the 50kDa or 70kDa regions (Fig. 4-1b, lane 2), and non-immune rabbit serum also gave a negative result (Fig. 4-1b; lane 1). There was found to be some temporal variation in the expression of cross-reactive proteins. The 70kDa band was present throughout the period of development studied, from day 1 (stage 5) to day 6 (stage 29; Fig. 4-1a, lanes 3 to 8), while the expression of the 50kDa protein was not detectable until day 3 (stage 18; Fig. 4-1a, lane 5). The 70kDa band showed a higher intensity than the 50kDa band throughout these stages, suggesting that it is the more predominant of the two molecules expressed during this time. It is possible that the two molecules detected are distinct TNFα cross-reactive proteins, or, alternatively the 50kDa molecule could represent a proteolytic cleavage product of the 70kDa form.

Reactivity of individual tissues (Fig. 4-1b), showed that proteins of both sizes could be detected in the heart at day 4 (stage 23; lane 5), while in the brain (lane 3), limb bud (lane 4), notochord (lane 6), and lens (lane 7), the 50kDa band was either absent or very faint. However, by day 6 (stage 29), the expression of the 50kDa band had increased such that all tissues examined expressed proteins of both sizes. Six-day lens tissue is shown as an example of this increase in expression, (Fig. 4-1b, lane 8; compare with lane 7).

When 6 day (stage 29) embryo homogenates were exposed to various concentrations of fresh glycosidase F, the 70kDa band remained (Fig. 4-2, lanes 1-4), though the 50kDa band, previously seen at 6 days (stage 29; Fig. 4-1a, lane 8), was absent from all lanes, including that with 0 Units/ml glycosidase F (Fig. 2, lane 1). Furthermore, no 17kDa band was seen when using glycosidase F.

Homogenates of whole 6 day (stage 29) embryos were also blotted with the two monoclonal antibodies (Fig. 4-3), both of which detected the 70kDa band, as well as a band of 120kDa (Fig. 4-3, lanes 2 and 4). One of the monoclonal antibodies also recognised the 50kDa band detected by the polyclonal antibody (Fig. 4-3, lane 2). Pre-absorption of the monoclonal antibodies with recombinant TNFα abolished all labelling (Fig. 4-3, lanes 1 and 3).

When using the protease inhibitor buffer and the enzyme chemiluminescence (ECL) detection method, in addition to the 50kDa and 70kDa bands, a band was detected in homogenate of 5 day (stage 26) embryos, upon exposure of the X-ray film for 3 seconds, which appeared to be slightly less than 17kDa in size (Fig. 4-4a, lane 3). An approximately 17kDa band was also detected in day 1 (stage 5) embryos, when the film was exposed for 15 seconds (Fig. 4-4b, lane 2).

4.3.3. Immunocytochemical localisation

The polyclonal antibody and both monoclonal antibodies were used for immunocytochemical localisation. All of these antibodies gave similar results, and the sites of immunoreactivity were the same with each. In general, the polyclonal antibody gave the more intense reactivity, and the results are therefore illustrated by this antibody.

i. Gastrulation (stage 5) to day 2 (stage 12): Immunoreactivity was detectable during gastrulation stages of development (Fig. 4-5a), in the endoderm and mesoderm cell layers, but not in the overlying epiblast. The cells of the primitive streak did not stain, but the mesoderm cells emerging from this region appeared to acquire immunoreactivity soon after leaving the vicinity of the streak. At day 2, (Fig. 4-5c), staining was detected primarily in the ectoderm and in the endoderm, but was not apparent in the somitic or lateral plate mesoderm. Control sections (Fig. 4-5b), showed low levels of background staining.

ii. Day 3 (stage 18): At cranial levels (Fig. 4-6a), staining appeared most prominently in the cranial nerves, the brain neuroepithelium and the notochord. At the cervical region (Fig. 4-6b), labelling appeared with high intensity in the myotome. The onset of this myotomal staining could be seen in the trunk, where the myotome cells at the dorso-medial edge of the maturing somite were just beginning to advance beneath the overlying dermatome (Fig. 4-6c). At this level, the migrating myotome cells were positively labelled, whereas the dermatome was not. Control sections showed low background staining (Fig. 4-6d).

iii. Day 4 (stage 23): By this time staining intensity had increased in the myotome and notochord, as well as in the marginal zone of the neural tube, and in ventral nerves (Fig. 4-7a). Spinal ganglia had become intensely immunoreactive (Fig. 4-7d). Whereas labelling of the myotome was a consistent finding, there were occasional instances in which the sclerotome of the cervical region was immunoreactive (Fig. 4-7b), but we were unable to correlate the reactivity in this tissue with a particular stage or rostro-caudal level. As before, control sections were not reactive (Fig. 4-7c).

iv. Day 5 (stage 26): At this stage the distribution of immunoreactivity was similar to that at day 4, however, in the cervical region, myotome staining was diminished, while staining in the mesenchyme immediately adjacent to it was increased (Fig. 4-8a). In the trunk, staining remained high in the notochord, myotome, marginal zone of the neural tube, and in the ventral nerves (Figs. 4-8b and 4-8c).

v. Day 6 (stage 29): Immunoreactivity was now present throughout the marginal zone of the neural tube, whereas previously staining could be detected only in the dorsal and ventral aspects of the neural tube (Fig. 4-9a, compare with Fig. 4-8c). The notochord has become highly vacuolated by this time, and intense immunoreactivity was observed surrounding these vacuoles (Fig. 4-9b). In the limb buds immunoreactivity was not intense, but was observed in the non-chondrifying mesenchyme (Fig. 4-9c), and could be seen in the associated nerves.

vi. The lens: This tissue showed a particularly consistent and intense staining pattern. At day 3, the lens showed no immunoreactivity (Fig. 4-10a). However, by day 4, at the time that the lens fibres have begun to elongate and their chromatin has begun to condense, the lens was highly immunoreactive (Fig. 4-10b), and continued to be so throughout the rest of the stages examined. Controls at these stages showed little or no background staining (Fig. 4-10c).

4.3.4. Ultrastructural immunogold labelling

Pre-embedding immunogold labelling of cells of the gastrulating (stage 5) embryo using the polyclonal antibody, showed that gold particles were readily bound by the surfaces of the mesoderm cells (Fig. 4-11a), but infrequently bound to the surface of cells of the epiblast layer (Fig. 4-11b). Control sections, in which the antibody was replaced by normal rabbit serum, showed very low background labelling (Fig. 4-11c). Quantification of the number of gold particles bound to these cell surfaces (Table 3-1) showed that the mesoderm cells bound approximately 5 times as much gold as the epiblast cells, while binding by the latter did not differ significantly from the control. Since pre-embedding immunogold labelling was carried out, there was no labelling observed in the cell interiors.

4.4. Discussion

These results show that TNFα-cross-reactive proteins have a widespread distribution during the early development of the chick and provide circumstantial evidence for a role for them in a number of processes. Although there are some previous data showing that TNFα transcripts and protein expression can be detected at later stages of development (Ohsawa & Natori, 1989; Gendron *et al.*, 1991; Clough *et al.*, 1992), it is shown here that proteins cross-reactive with antibodies to TNFα are present at much earlier stages, and with a more widespread distribution, than previously suspected.

Using Western immunoblotting the presence of molecules, with TNFα-cross-reactivity in the early chick embryo, has been substantiated. Several of them are larger, at 50kDa, 70kDa

and 120kDa, than both the 17kDa monomeric secreted form from monocytes and macrophages (Sprang & Eck, 1992), and the 26kDa integral membrane form (Kriegler, 1988; Luettig, 1989; Sprang & Eck, 1992), although using the protease inhibitor buffer (Jaskoll *et al.*, 1994) a protein that is approximately 17kDa in size has been detected on day 1 (stage 5) and day 5 (stage 26) of development. This is the first demonstration of a chicken TNFα-cross-reactive protein that is close to the recognised molecular weight for TNFα; i.e. 17kDa. The fact that the proteins detected in the present work are specifically recognised by several different antibodies against TNFα, including two monoclonal antibodies, supports the contention that each of them is in fact TNFα-related, and not due to the presence of similar epitopes on unrelated molecules. Indeed, there are several reports referring to molecules exhibiting TNF-like activities, which are of similar size to the 50kDa and 70kDa molecules detected here. Liu *et. al.* (1987; 1989) have reported the detection of cytosolic as well as membrane bound cytotoxins, which are immunologically related to TNFα, in murine cytotoxic T-lymphocytes, while Kinkhabwala *et al.* (1990) have detected expression of similarly sized molecules, (50-60 kDa and 70-80 kDa), on the surface of activated T-cells. The exact nature of the high molecular weight proteins detected here remains uncertain, but they could represent avian members of the TNF-ligand family (see Chapter 2).

However, using ultrastructural immunogold labelling, TNFα immunoreactivity has been shown to be associated with the cell surface of mesoderm cells. Liu *et al.* (1989) also demonstrated a cell surface localisation and it is therefore supposed that at least some of the antigen is membrane bound. The expression of TNFα-like proteins on the surface of embryonic cells may be advantageous in that it would provide a means of localising the effects of TNFα during development, just as membrane bound TNFα does in the immune system (Kinkhabwala, 1990).

These results agree with those of Gendron *et al.* (1991), who also detected a TNFα-like molecule of 50kDa during chick nervous system development (though at later stages than those investigated here) and of Ouwe-Missi-Oukem-Boyer *et al.*, (1994) who detected a 53 kDa TNFα immunoreactive molecule in mollusc haemocytes. The 50kDa protein found here may be similar to the 50kDa TNF-related cytokine recently characterised by Goodwin *et al.*,

(1993), who suggest that N-linked glycosylation might account for the higher than expected M_r of that molecule. However, exposure of embryonic homogenate to glycosidase F did not reduce the M_r in a similar immunoblotting experiment (Fig. 4-2).

The work described in this Chapter provides the most detailed spatio-temporal analysis to date regarding the distribution of TNFα-cross-reactive protein expression during early embryonic development. TNFα immunoreactivity has been detected at various stages of embryonic development, which are described on the following pages, at sites of major morphological re-organisation, several of which are known to undergo programmed cell death.

4.4.1. From gastrulation (stage 5) to two days (stage 12)

Staining was first detected at 1 day (stage 5) of development, in the gastrulating embryo, in the cells of the mesoderm and endoderm. Gastrulation is a pivotal event in early embryogenesis in which the two layered embryo becomes three layered by passage of cells from the upper epithelial layer (epiblast) through a specialised region known as the primitive streak (Sanders, 1986). This is the first major morphogenetic re-organisation occurring during development, and results in the formation of the mesoderm.

What might be the role of TNFα at this early stage of development? It has been shown by Takahashi *et al.* (1992) that TNFα and IL-1ß have the ability to induce activin-A gene expression in a bone marrow cell line. Activin-A is a TGFß-like molecule belonging to a family of proteins implicated in induction of axial/dorsal mesoderm in both amphibians (reviewed by New & Smith, 1990), and amniotes (Mitrani, *et al.* 1990; reviewed by Stern, 1992). Thus, TNFα-like molecules may be able to up-regulate activin expression during early stages of development.

Also, recent work reveals that TNFα can stimulate lung fibroblast hyaluronan synthesis (Sampson *et al.*, 1992). Hyaluronan is considered to have an important role during gastrulation in keeping cells separated, thereby discouraging premature aggregation, adhesion, and differentiation (Toole, 1991) and in promoting cell locomotion (Turley, 1989). Proteins with TNFα-like activity could therefore promote cell locomotion during gastrulation through

up-regulation of hyaluronan synthesis, since the latter is known to be the predominant ECM molecule present at this time (Fisher and Solursh, 1977).

Alternatively, TNFα-like molecules could be involved in programmed cell death at these stages. Sanders and Wride (1995) have reviewed the literature pertaining to the incidence of cell death in early embryos, and have identified several stages where it may occur. Of particular relevance to the present study is the detection of cell death in the epiblast/ectoderm during mesoderm formation, and in the primitive endoderm at the time of head process formation. Although TNFα reactivity has not been detected in the ectoderm at this stage, the latter case is relevant to the observations described here, since TNFα immunoreactivity has been detected in the endoderm. About 50% of the primary endoderm cells in the ventral mid-line die during this time (Poelmann, 1981a,b; Lawson *et al.*, 1986). This work has been carried out on mouse embryos, but cell death has been identified in all parts of the chick blastoderm at the primitive streak and head process stages, both in the area pellucida and area opaca (Bellairs, 1961; Sanders *et al.*, 1996).

During the 2nd day of embryonic development (stage 12), TNFα immunoreactivity has been detected only in the ectoderm, endoderm, and mesenchymal tissue, where it could presumably have a role in tissue remodelling, as suggested by Vlassara *et al.* (1988) for adult tissue. However, expression reappears in several tissues undergoing morphogenetic re-organisation at 3-4 days of development, such as myotome, sclerotome, notochord, lens, and nervous tissue.

4.4.2. The myotome

TNFα immunoreactivity was very prominent in the myotome component of the somitic mesoderm at all stages examined. Evidence for cell death in the myotome is limited, although in amphibians, Chung *et al.* (1989) have shown that cells of the cranial myotomes appear to possess an autonomous programme for cell death. However, TNFα immunoreactivity is not confined to the cranial myotomes in the present study implying a wider role for proteins with TNFα-like activity in the differentiation of muscle, as suggested by Miller *et al.* (1988).

4.4.3. The notochord

The notochord plays an important role in early embryogenesis with roles in somite differentiation, induction of the neural plate, and in the cellular organisation of the neural tube, (Placzek *et al.* 1990). Later, the notochord becomes progressively more vacuolated, and begins to degenerate, eventually contributing to the spinal column. Even before overt deterioration, rod shaped nuclei, which are possibly degenerating nuclei of non-vacuolated cells, can be seen at 6 days (stage 29) of development (Kuhlenbeck, 1930), while by day 10 (stage 36) the gross degeneration of the notochord can be readily observed (Williams, 1942). This degeneration includes nuclear disintegration and a decrease in the size of the vacuoles until a day later only a small amount of notochordal tissue remains. Here, TNFα immunoreactivity is detected in the notochord from 3 days (stage 18) of development onwards. This precedes the appearance of the marked morphological changes, and implies that TNFα may be a prerequisite for these processes, although the presence of immunoreactivity throughout this period of notochordal differentiation suggests a continued requirement for TNFα in the notochord during this time.

4.4.4. The lens

The presence of TNFα reactivity in the lens is particularly interesting in view of the well characterised degradative processes occurring in this tissue during early development (reviewed by Piatigorsky, 1981; Counis *et al.*, 1989). Degeneration of cellular organelles and DNA occurs in the fibre cells, preceding the co-ordinated deposition of crystallin proteins, which are the major structural components of the mature lens. This degeneration has already begun at 8 days of development (Modak & Perdue, 1970) and the condensed chromatin exhibited by the lens fibre nuclei during this process is highly characteristic of the morphological changes that accompany, and help to define, apoptosis (see Chapter 6). It is suggested here that the presence of TNFα-cross-reactivity in the lens fibres, preceding these degradative events, is consistent with role for TNFα related proteins in this process. Thus, it is

possible that these factors have similar roles in both lens fibre cells and in the notochord, in the initiation and the progression of the degenerative events.

4.4.5. The nervous system

TNFα immunoreactivity has previously been detected in brain neuroepithelium of the chick at day 8 (stage 34) by Gendron *et al.* (1991). Here, TNFα immunoreactivity is detected in nervous tissue from day 3 (stage 18) of development in the outer neuro-epithelial layer of the brain, and in the marginal zone of the neural tube. These results are also in agreement with those of Gendron *et al.* (1991) in that TNFα reactivity has been identified in the ventral nerves extending from the neural tube as well as cranial nerves, while dorsal root ganglia are consistently negative. Moreover, spinal and cranial ganglia are also intensely labelled at these early stages.

Programmed cell death has a well recognised role in neuronal development (Oppenheim, 1991), occurring in almost all parts of the nervous system during the phase of axon outgrowth and the establishment of peripheral connections (Snow, 1987). Chick spinal ganglia undergo massive degeneration between 4.5 days (stage 25) and 7.5 days (stage 32) of development (Hamburger, 1992), and in the motor columns of the chick embryo about 40% of the motoneurons die between days 5 and 9 (Stages 27 and stage 35) of development (Hamburger, 1975; 1992). In the present study, TNFα immunoreactivity is detected in spinal ganglia on day 4 (stage 23) of development and throughout the neural tube, although staining is most intense in the marginal zone (which does not contain cell bodies). Therefore, the detection of TNFα-cross-reactivity corresponds with some areas of the chick nervous system that are undergoing programmed cell death. However, it should be emphasised that TNFα immunoreactivity does not correspond exclusively to areas undergoing cell death. This suggests, once again, that TNFα could have other potential roles in nervous system development distinct from any effects it might have on cell death; e.g. on proliferation and/or differentiation, as suggested in Chapter 3.

4.4.6. Concluding comments

In this Chapter, a spatio-temporal analysis of the distribution of TNFα immunoreactivity during development of the early chick embryo has been provided. There is 71% primary sequence conservation of TNFα across 9 mammalian species Sprang & Eck, (1992). This is a high degree of homology and implies a high degree of evolutionary conservation. It has been suggested that factors controlling fundamental differentiative events will be highly conserved whereas factors with a lesser degree of homology will have evolved to carry out more specialised functions in particular species (Lee, 1992). In view of the widespread detection of TNFα reactivity in the present study, and the implied role of TNFα-like proteins in a number of important differentiative processes, it is possible that TNFα-like proteins may fall into the former category suggesting at least an important, if not a fundamental, role for them in development.

The precise nature of this role awaits further study, but in the present work TNFα immunoreactivity has been detected in several tissues that are undergoing ECM remodelling; e.g. the migrating cells of the mesoderm and the sclerotome, and in several tissues that are undergoing programmed cell death or nuclear degeneration at the stages investigated; e.g. the notochord, parts of the nervous system, and the lens. Kerr *et. al.*, (1987) have pointed out that apoptosis is a phylogenetically primitive process, and these authors hypothesised that during evolution the immune system may have made use of pre-existing cellular mechanisms (such as those in embryonic cells), for regulating cell death, by acquiring an ability to produce proteins such as TNFα, for example, which have the ability to mediate apoptosis. Thus, it is possible that other molecules, which hitherto have known roles only in the immune system and in inflammatory tissue remodelling, may have unsuspected roles during embryonic development.

TABLE 4-1. Immunogold-labelling of cell surfaces of the stage 5 embryo.

Gold particles per 10μm of linear cell surface, ± estimated SEM, n = 10.

	Mesoderm	Apical epiblast
Anti-TNFα	19.2 ± 1.7	4.2 ± 0.7*
Rabbit serum.	2.0 ± 0.8*	0
PBS	0.4 ± 0.2*	0

* significantly different from the binding of anti-TNFα to the surface of mesoderm cells, ($p = 0.05$).

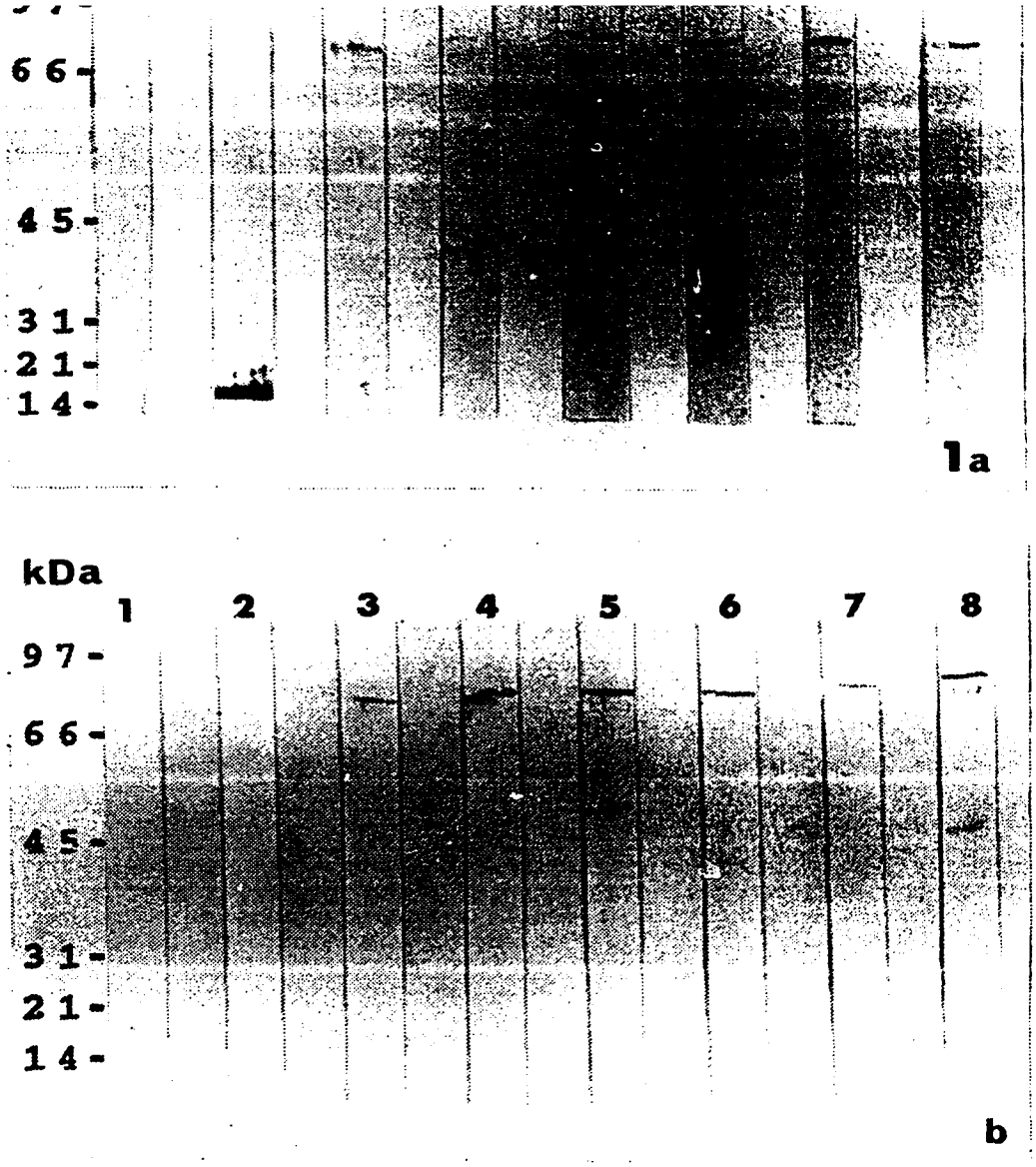

Figure 4-1a. Western blot analysis demonstrating the specificity of polyclonal mouse TNFα antiserum for recombinant mouse TNFα, and for TNFα cross-reactive proteins of approximately 50kDa and 70kDa, present in the early chick embryo. Lane 1: recombinant mouse TNFα probed with polyclonal anti-mouse TNFα pre-absorbed with mouse TNFα; Lane 2: recombinant mouse TNFα probed with polyclonal anti-mouse TNFα; Lane 3: homogenate from whole 1 day (stage 5) embryos; Lane 4: 2 day (stage 12) embryos; Lane 5: 3 day (stage 18) embryos; Lane 6: 4 day (stage 23) embryos; Lane 7: 5 day (stage 26) embryos; Lane 8: 6 day (stage 29) embryos.

Figure 4-1b. Western blot analysis demonstrating the variation of expression of TNFα cross-reactive proteins in different tissues during early chick embryo development. Lane 1: serum; Lane 2: homogenate from whole 6 day (stage 29) embryos probed with polyclonal anti-mouse TNFα preabsorbed with recombinant mouse TNFα; Lane 3: 4 day (stage 23) brain; Lane 4: 4 day (stage 23) limb buds; Lane 5: 4 day (stage 23) heart; Lane 6: 4 day (stage 23) notochord; Lane 7: 4 day (stage 23) lens; Lane 8: 6 day (stage 29) lens.

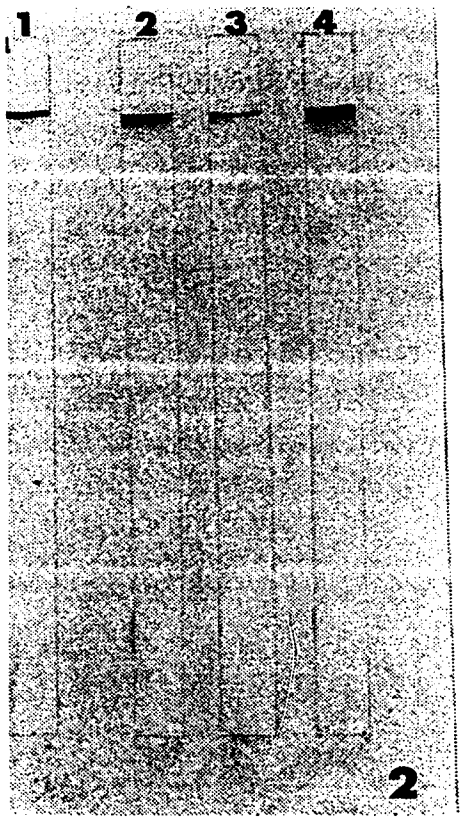

Figure 4-2. Western blot analysis demonstrating that glycosidase F is ineffective, in revealing TNFα immunoreactive bands of reduced molecular weight, when incubated with 6 day (stage 29) embryo homogenate. All lanes probed with polyclonal anti-mouse TNFα. Lane 1: 0 Units/ml glycosidase F; Lane 2: 10 Units/ml glycosidase F; Lane 3: 20 Units/ml glycosidase F; Lane 4: 30 Units/ml glycosidase F.

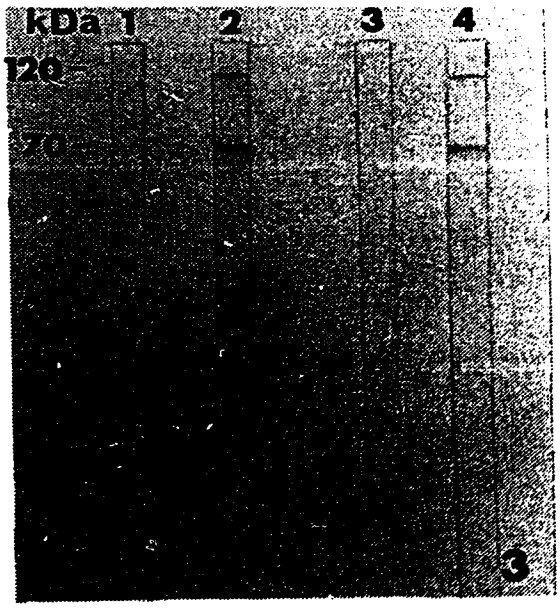

Figure 4-3. Western blot analysis demonstrating the specificity of the monoclonal anti-mouse TNFα antibodies for TNFα-cross-reactive proteins in homogenate of whole 6 day embryos. Lane 1: Monoclonal rat anti-mouse TNFα, clone MP6-XT22, pre-absorbed with mouse TNFα; Lane 2: Monoclonal rat anti-mouse TNFα, clone MP6-XT22; Lane 3: Monoclonal rat anti-mouse TNFα, clone MP8-XT22, pre-absorbed with mouse TNFα; Lane 4: Monoclonal rat anti-mouse TNFα, clone MP8-XT22.

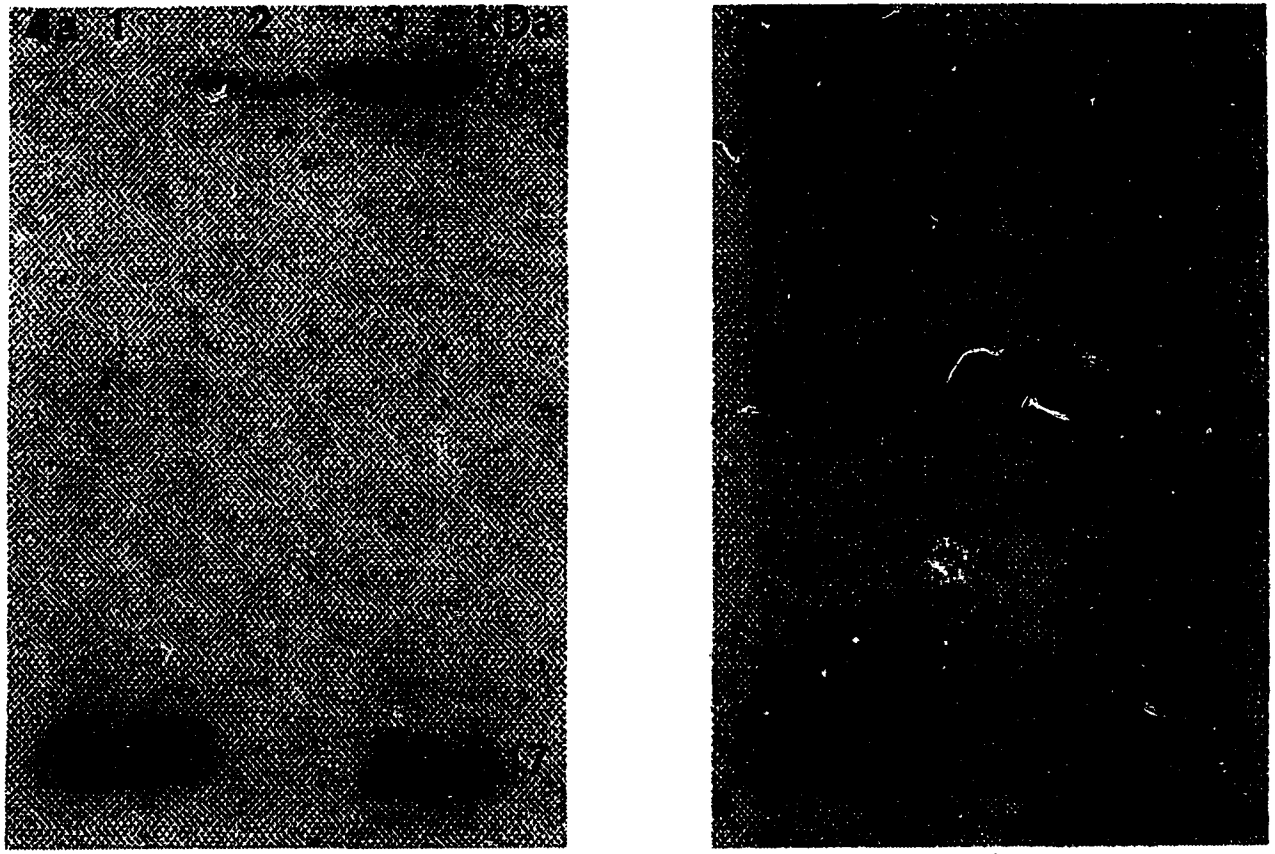

Figure 4-4. Western blot analysis using protease inhibitor buffer to homogenise day 1 (stage 5) and day 5 (stage 26) embryos and using chemiluminescence to detect bands. (a). (X-ray film exposure time ≈ 3 seconds). Lane 1: recombinant mouse TNFα probed with polyclonal anti-mouse TNFα; Lane 2: day 1 (stage 5) embryo homogenate probed with polyclonal anti-mouse TNFα; Lane 3: day 5 (stage 26) embryo homogenate probed with polyclonal anti-mouse TNFα. **(b).** Same Western blot as Figure 4-4a, but X-ray film exposed for 15 seconds.

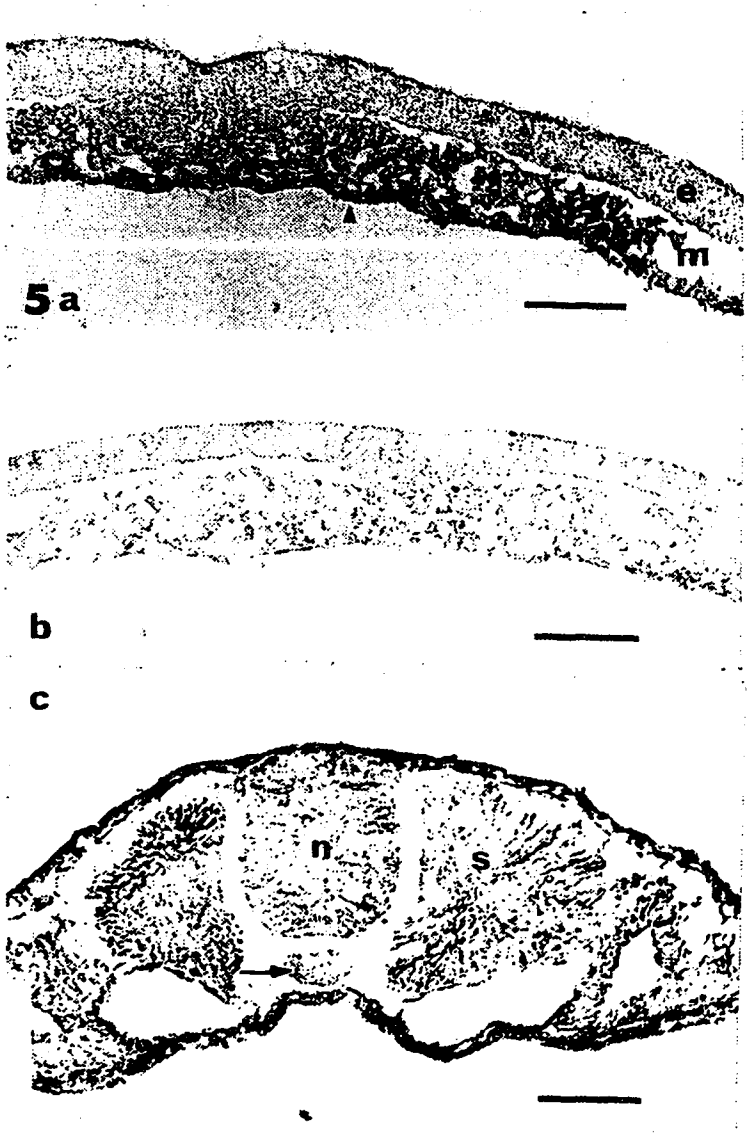

Figure 4-5. Immunocytochemical localisation of TNFα at days 1 (stage 5) and 2 (stage 12). (a). Day 1 (stage 5). Immunocytochemical localisation of TNFα during gastrulation. Section through a 24 h, primitive streak-stage embryo, showing staining in the mesoderm (m) and endoderm (arrowhead), but not in the epiblast (e). Bars = 50μm. **(b).** Day 1 (stage 5). As Fig. 4-5a, but incubated with non-immune rabbit serum. **(c).** Day 2 (stage 12). Immunoreactivity is evident in the ectoderm and endoderm, but not in the neural tube (n) or notochord (arrow). The mesoderm of the somites (s) is generally non-reactive at this stage.

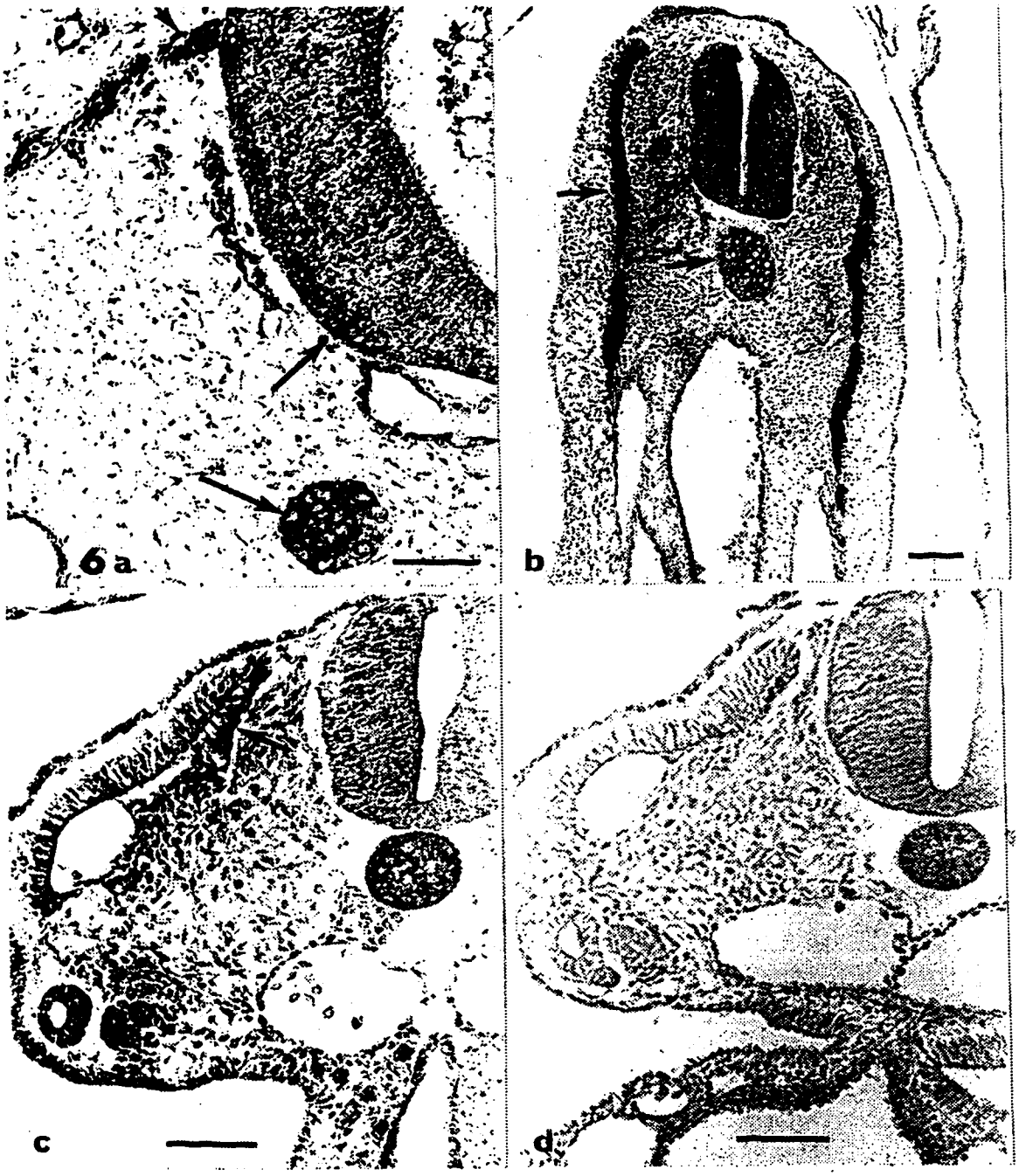

Figure 4-6. Immunocytochemical localisation of TNFα at day 3 (stage 18). (a). Day 3 (stage 18). Staining is visible in the neurocpithelium of the brain, the cranial nerve, and the notochord (arrows). Bars = 50μm. **(b).** Day 3 (stage 18). Section through the cervical region showing prominent staining in the notochord and myotome (arrows). **(c).** Day 3 (stage 18). Section through the trunk showing staining in the forming myotome (arrow). **(d).** Day 3 (stage 18). As Fig. 4-6c, but incubated with non-immune rabbit serum.

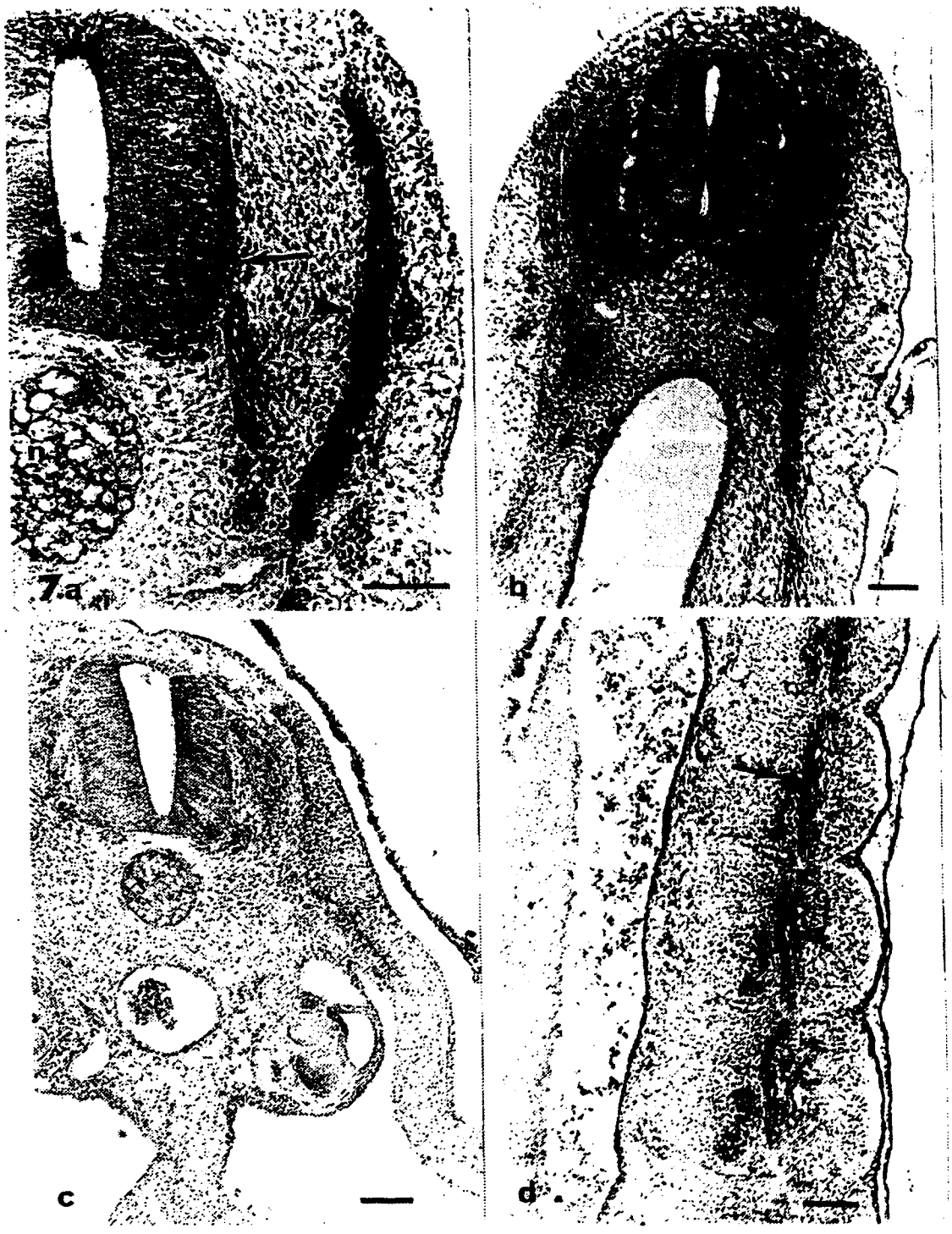

Figure 4-7. Immunocytochemical localisation of TNFα at day 4 (stage 23). (a). Day 4 (stage 23). Staining is present in the myotome (arrowhead), notochord (n), marginal zone of the neural tube (arrow) and in the ventral nerve. Bars = 50μm. **(b).** Day 4 (stage 23). Section through cervical region showing staining in the sclerotome. **(c).** Day 4 (stage 23). As Fig. 4-7a, but incubated with non-immune rabbit serum. **(d).** Day 4 (stage 23). Mid-sagittal section showing staining in spinal ganglia (arrow) and myotomes (arrowhead).

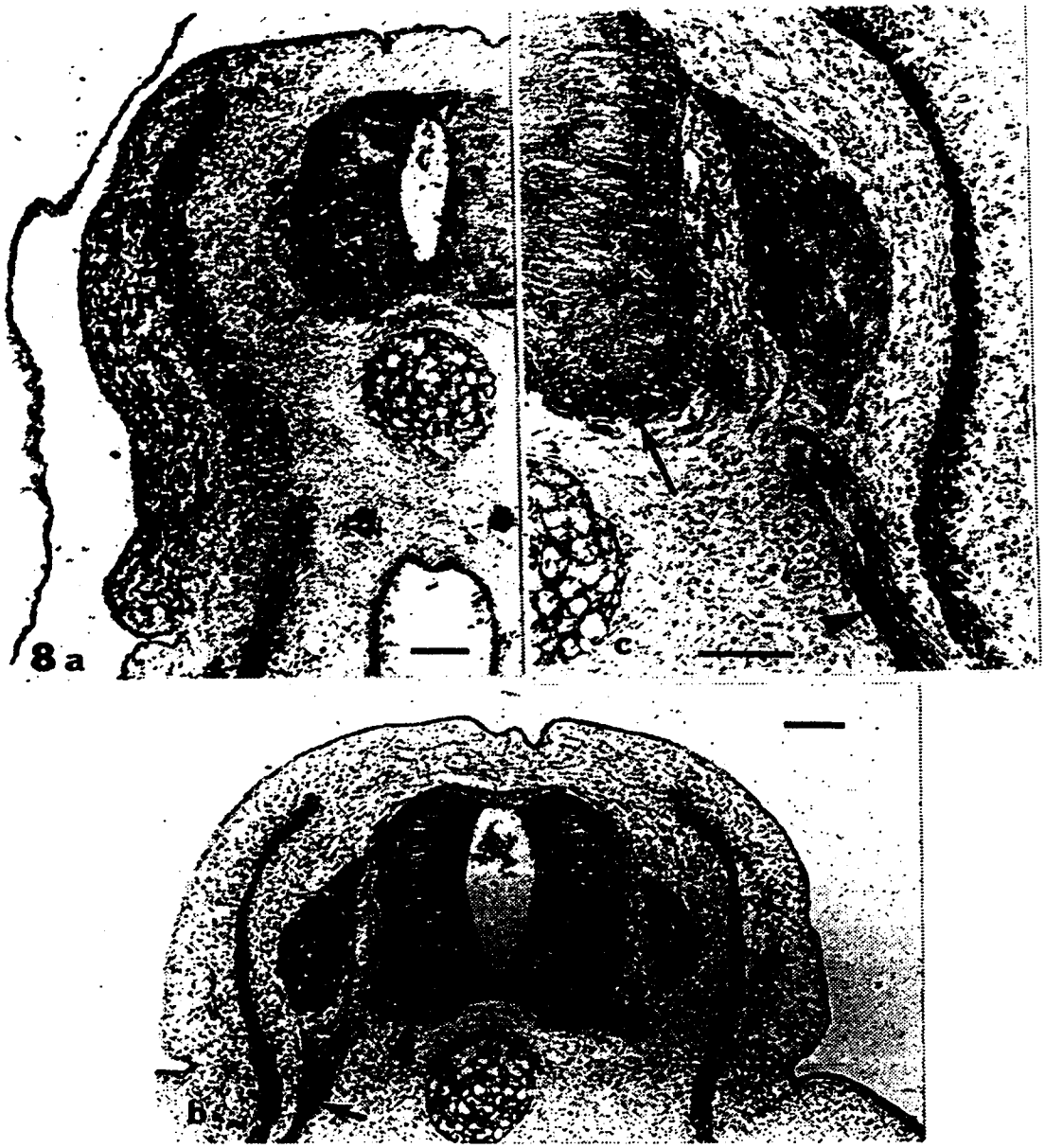

Figure 4-8. Immunocytochemical localisation of TNFα at day 5 (stage 26). (a). Day 5 (stage 26). Section through the cervical region showing staining in the mesenchyme (m) lateral to the somite, the marginal zone of the neural tube (arrow), and the notochord (n). Bars = 50μm. (b). Section through the trunk showing staining in the myotome, ventral nerve (arrow), marginal zone (arrowheads) and notochord. (c). Day 5 (stage 26). As Fig. 4-8b, showing details of the immunoreactivity in the marginal zone of the neural tube (arrow), the myotome and ventral nerve (arrowhead).

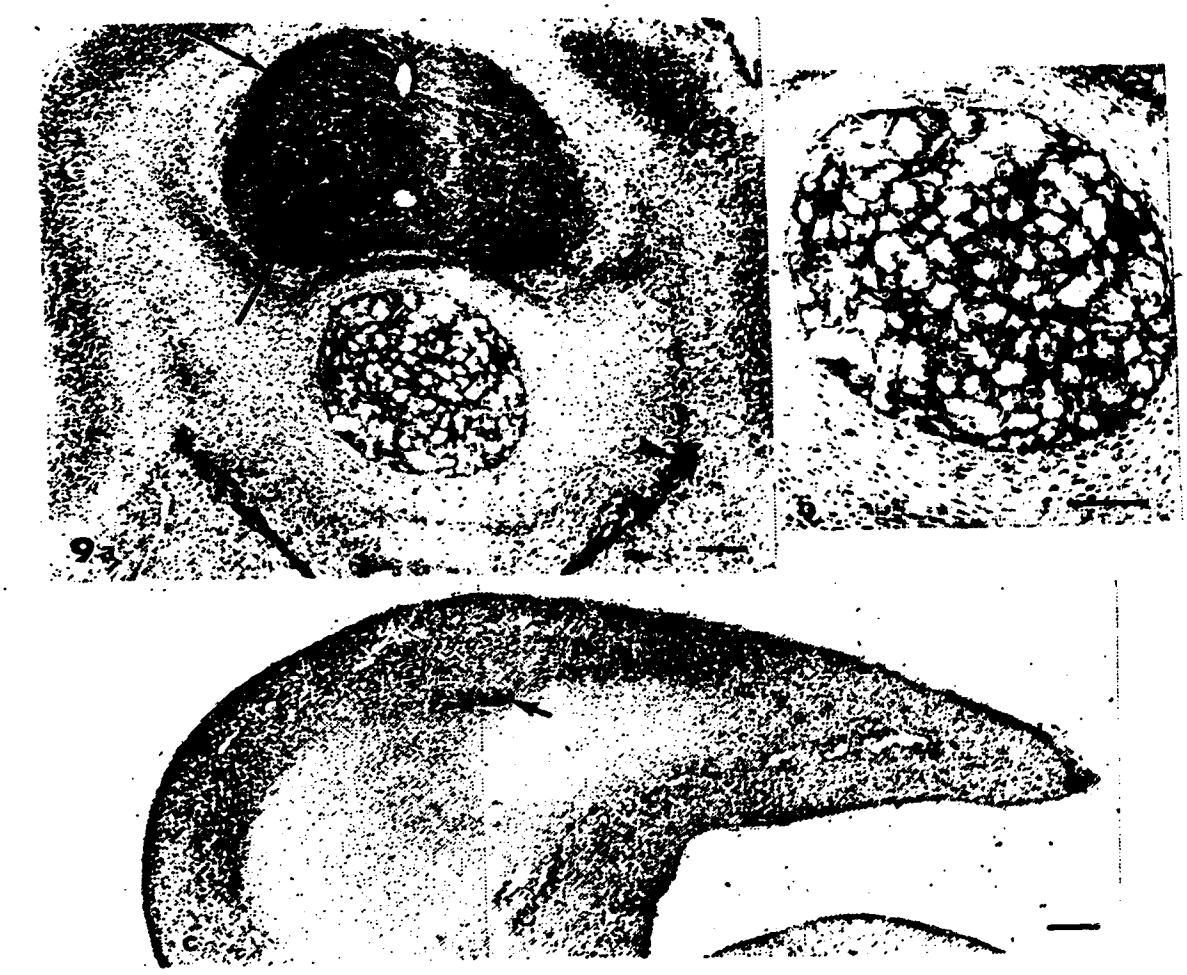

Figure 4-9. Immunocytochemical localisation of TNFα at day 6 (stage 29). (a). Day 6 (stage 29). Section through the nerve cord showing staining in the marginal zone (arrows), notochord and ventral nerve (arrowhead). Bars = 50μm. **(b).** Day 6 (stage 29). The notochord, showing labelling surrounding the vacuolated cells. **(c).** Day 6 (stage 29). Limb bud, showing staining in the peripheral, non chondrogenic, mesenchyme (m), and in nerves (arrow).

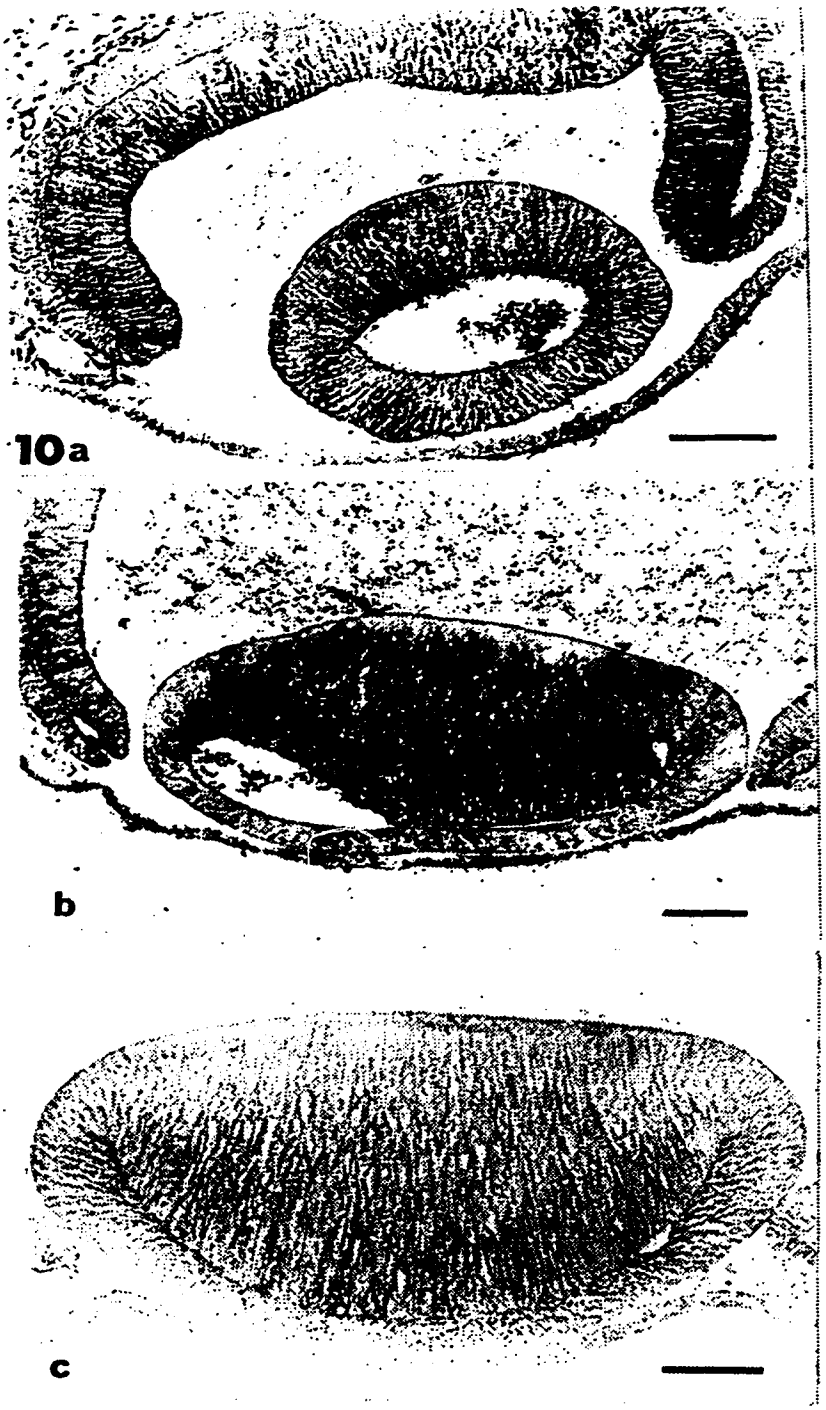

Figure 4-10. Immunocytochemical localisation of TNFα in the lens. (a). Lens, day 3 (stage 18). At this stage the lens is non-reactive. Bars = 50μm. (b). Lens, day 4 (stage 23). Staining is present in the lens fibres, but not in the lens epithelium. (c). Lens, day 4 (stage 23). As Fig. 4-10b, but incubated with non-immune rabbit serum.

Figure 4-11. Ultrastructural immunogold labelling of the surface of cells from day 1 (stage 5) embryos using a polyclonal anti-TNFα antibody. (a). Day 1 (stage 5). Ultrastructural immunogold labelling of the surface of a mesoderm cell using the polyclonal anti-TNFα antibody. Gold particles are associated with the surface of these cells. Bars = 250nm. **(b). Day 1 (stage 5).** As Fig. 4-11a, showing the apical surface of the epiblast cell layer. Gold particles are seen very infrequently on these cells (see Table 4-1). **(c). Day 1 (stage 5).** As Fig. 4-11a, showing the surface of a mesoderm cell. In this case, the antibody was replaced with normal rabbit serum. Background labelling (arrow) is very low (see Table 4-1).

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CHAPTER 5

DISTRIBUTION OF TNFα-LIKE PROTEINS CORRELATES WITH SOME REGIONS OF PROGRAMMED CELL DEATH IN THE CHICK EMBRYO[1]

5.1. Introduction

It is well established that normal embryonic development is accompanied by a co-ordinated pattern of cell deletion (Snow, 1987; Ellis *et al.*, 1991; Sanders and Wride, 1995). This "programmed cell death" is seen very early in development (Bellairs, 1961; Sanders *et al.*, 1996a), and has a vital role in the differentiation of many tissues and organs, including: the limb buds (Hinchliffe, 1981), the nervous system (Oppenheim, 1991; Barinaga, 1993), and the heart (Pexieder, 1975). Programmed cell death is frequently characterised morphologically by apoptosis, which is identifiable by early nuclear compaction, cytoplasmic condensation and blebbing of both nuclear and plasma membranes (reviewed by Kerr *et al.*, 1987).

Previously, programmed cell death could only be detected *in situ* using vital dyes taken up through the perforated membranes of dying cells (Saunders *et al.*, 1962, Jeffs and Osmond, 1992); however, loss of membrane integrity is a relatively late event in cell death (Vaux, 1993). Alternatively, cells undergoing apoptosis can be identified using the purely morphological criteria described above (Kerr *et al.*, 1987), but dying cells are removed very rapidly during programmed cell death, and are not easily identifiable in tissue sections.

A technique that allows identification of cells undergoing apoptosis *in situ* is that of terminal deoxynucleotide transferase mediated dUTP-biotin nick-end labelling (TUNEL), which was recently re-discovered by Gavrieli *et al.* (1992) after Iseki and Mori (1985), Iseki (1986), and Modak and Bollum (1970) had described it, or very similar techniques, previously. This method is based on the fact that apoptosis is usually accompanied by fragmentation of nuclear DNA between nucleosomes by endogenous endonucleases (Gerschenson and Rotello, 1992). Although several recent studies have revealed that the morphology associated with

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[1] *A version of this Chapter has been published. Wride M A, Lapchak P H, Sanders E J. (1994) Distribution of TNFα-like proteins correlates with some regions of programmed cell death in the chick embryo. Int. J. Dev. Biol. 38: 673-682.*

apoptosis can occur without DNA fragmentation in some instances (Cohen *et al.*, 1992; Tomei *et al.*, 1993; Zakeri *et al.*, 1993), this fragmentation remains an essential feature of programmed cell death in many cases, and may be an early event in the cell death pathway. TUNEL allows detection of cells undergoing DNA fragmentation, by incorporation of labelled nucleotides at free 3'-OH ends of nicked DNA using terminal deoxynucleotide transferase (TdT). These transient dying cells can therefore be detected cytochemically in tissue sections. Furthermore, if cells are both TUNEL-positive and display characteristic morphological features, they can then be designated as truly apoptotic.

It has been suggested that several homeobox genes have a role in cell death during chick limb development (Coelho *et al.*, 1993) and the expression of the homeobox gene *msx* correlates with apoptosis in the neural crest of the chick hindbrain (Graham *et al.*, 1993). Proto-oncogenes may also be involved, as suggested by the recent report of *c-fos* expression preceding programmed cell death *in vivo* (Smeyne *et al.*, 1993). In the central core of mouse embryo limb buds, apoptosis may be accompanied by a transient increase in tissue transglutaminase expression (Jiang and Kochhar, 1992), while the testosterone-repressed prostate message-2 gene accompanies programmed cell death of interdigital tissue (Buttyan *et al.*, 1989). The product of the polyubiquitin gene has also been shown to accompany programmed cell death in some cases (Schwartz, 1991). However, the identity of many of the proteins expressed during programmed cell death *in vivo* remains undetermined. This is particularly true of the putative "suicide" or "killer" proteins that are proposed to actually initiate the events of programmed cell death.

In Chapter 4, the existence of a developmentally regulated expression of TNFα-cross-reactive proteins, during the early development of the chick embryo, was reported. Considering the ability of TNFα to instigate DNA fragmentation and apoptosis in sensitive cells (Larrick and Wright, 1990; Wright *et al.*, 1992) and the developmental distribution of TNFα-cross-reactive proteins, it was speculated that one of the roles of proteins with TNFα-like activity in embryos could be the initiation of programmed cell death at specific sites and times during development.

In the studies described in this Chapter, TUNEL was used to detect cells undergoing programmed cell death and, in some locations, a close correlation was noted between the expression of TNFα-cross-reactive proteins and cells that are undergoing DNA fragmentation detected by TUNEL. These tissues included: the ventral horns of the neural tube, spinal nerves, paravertebral ganglia, parts of the myotome, mesenchyme of the body wall, and the mesonephros. It is also shown that chick embryo homogenate possesses endogenous TNFα-like cytotoxic activity, which could be reduced when homogenate was incubated with neutralising antibodies to TNFα. Thus, TNFα- cross-reactive proteins could be involved in programmed cell death in some tissues during early chick embryo development.

5.2. Materials and methods

5.2.1. Preparation of Embryos for Immunocytochemistry and TUNEL

White Leghorn hens' eggs were incubated at 37°C for 3 to 6 days (Stages 18-29; Hamburger and Hamilton, 1951). The embryos were removed from their yolk and rinsed and handled in Tyrode's saline. Any morphologically abnormal embryos were discarded and normal embryos were fixed with 4% paraformaldehyde overnight at 4°C, washed in phosphate buffered saline (PBS; pH 7.4), dehydrated through a graded series of ethanol, cleared in xylene and embedded in paraffin wax. Blocks were sectioned at 5μm thickness.

5.2.2. Immunocytochemistry

Immunocytochemistry for TNFα was carried out as described previously (see Chapter 4) using the alkaline phosphatase based AS/AP® kit (BioCan Scientific Inc.), and two rat monoclonal antibodies to mouse TNFα. Clone MP6-XT22, was obtained from either Endogen Inc. or abV ImmuneResponse Inc., while clone MP8-XT22 was obtained from UBI Inc. These antibodies were used at a dilution of 1:20, and detected with the AS/AP® system in which the second antibody was replaced by biotinylated rabbit anti-rat IgG (Dimension Laboratories Inc.) diluted 1:50 in TBS (pH 7.6) plus blocking agent (supplied in the kit). Antibody preabsorbed with mouse TNFα was used as a control, as described previously (see Chapter 4).

5.2.3. TUNEL

Incorporation of biotinylated nucleotides at free 3'-OH ends of DNA in tissue sections was carried out as described by Gavrieli *et al.* (1992) with modifications according to Wijsman *et al.* (1993). Tissue sections were cleared in Hemo-De (2 x 5mins), rehydrated through a graded series of alcohol, and were finally immersed in distilled de-ionised water (DDW). Sections were then incubated for 20 mins in 2 x saline sodium citrate (SSC) buffer (0.3M sodium chloride, 30mM sodium citrate, pH 7) at 60°C, washed in DDW, and immersed in proteinase K buffer (10mM Tris-HCl, pH 8) for 10 mins at room temperature. This was followed by incubation with 20µg/ml of freshly prepared proteinase K (Sigma), for 15 mins at room temperature, and a final wash in DDW. Endogenous peroxidase was inactivated by covering the sections with 2% H_2O_2 in DDW with 0.5% Tween 20 for 10 mins at room temperature. Sections were then incubated in 10 x TdT buffer, pH 7.2 (30mM Trizma base, 140mM sodium cacodylate, 1mM cobalt chloride) for 5 mins at room temperature. The reaction mixture was prepared as follows, allowing 75µl for each slide: DDW, 62.25µl; 10 x TdT buffer, 7.5µl; 5.25µl biotin-4-dUTP stock (Sigma; 0.5mM in 10mM TRIS-HCl); and 1.5µl TdT (10units/µl; Boehringer Mannheim). The sections were exposed to reaction mixture and then carefully covered with a glass cover slip and incubated at 37°C for 1-1.5 hrs in a humid chamber. The reaction was terminated by immersion of the slides in 2 x SSC at room temperature for 15 mins. Following two further washes in DDW and PBS (pH 7.4), the sections were covered in 3% skimmed milk (SM) in PBS with 0.5% Tween 20 for 15 mins to block non-specific binding. Following this, excess SM was removed and sections were incubated with Extra-avidin-peroxidase® (Sigma), diluted 1:50 in 3% SM in PBS with Tween 20, for 30 mins at room temperature. Slides were then washed in PBS, and sections were stained using the (3-amino-9-ethyl carbazole) AEC procedure (Pierce). Stock AEC solution was prepared by dissolving AEC in di-methyl formamide (DMF) to a final concentration of 4mg/ml. Prior to the colour reaction, 0.67ml of AEC stock was added to 10ml of 0.1M sodium acetate buffer (pH 5.2) and 10µl of 30% H_2O_2 was then added. This solution was filtered and applied to the sections and after 20 mins the colour reaction was complete. The slides were then washed in PBS and mounted in Crystal Mount® (Fisher Scientific Inc.).

Positively stained nuclei were coloured intensely red. In later experiments, TUNEL was performed using biotin-16-dUTP (Sigma). This was available at a concentration of 0.3mM, therefore the volumes in the reaction mixture were modified accordingly. Identical results as with biotin-4-dUTP were obtained with biotin-16-dUTP.

In addition, both positive and negative controls were performed. In the negative controls, either the bio-4-dUTP stock or the TdT was omitted from the reaction mixture. For positive controls, sections were treated with DNase 1 buffer (30mM Trizma base, 140mM sodium cacodylate, 4mM magnesium chloride, 0.1mM dithiothreitol) for 5 mins following the quenching of endogenous peroxidase, incubated with fresh DNase 1 (1μg/ml; Boehringer Mannheim) in buffer for 10 mins at room temperature, and then washed in DDW. Processing of the sections was then continued as described above. In negative control slides, no staining was observed in any of the nuclei and the light pink background colour was identical to that observed in experimental sections. In positive control sections treated with DNAse 1, every nucleus was stained intensely red (not shown).

5.2.4. Examination of sections for TUNEL-positive cells and TNFα-CRP expression

In order to investigate whether a correlation might exist between the expression of TNFα-cross-reactive proteins and the presence of programmed cell death in particular tissues, TUNEL was carried out on sections from a region closely adjacent to those that had been stained with the anti-TNFα antibodies immunocytochemically. These sections were examined and correlations were noted between the sites of expression of TNFα-cross-reactive proteins and TUNEL-positive cells in specific tissues. The results obtained were shown to be consistent by the use of sections from 3-5 different embryos at each stage of development studied.

5.2.5. TUNEL/anti-TNFα double labelling

A double labelling procedure was also used to investigate possible correlations between the expression of TNFα-cross-reactive proteins and the presence of DNA strand breaks indicative of programmed cell death, detected by TUNEL. Using the monoclonal antibodies,

sections that had been previously processed for TUNEL were immunostained using the AS/AP® kit. However, the chromogenic substrate supplied in the kit was replaced by AP colour development reagent consisting of 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (BCIP/NBT; BioRad) so that immunostaining by TNFα antibodies could be visualised as a light blue colour, which contrasted well with the intensely stained red nuclei that were detected by TUNEL. Immersion of sections in SSC buffer, and subsequent digestion with proteinase K did not affect the TNFα immunostaining.

5.2.6. Preparation of embryos for the L929-8 TNF bioassay.

White leghorn hens' eggs were incubated at 37°C for 3 days (stage 18; Hamburger & Hamilton, 1951). Fifty embryos were removed from their yolk and rinsed and handled in sterile millipore-filtered PBS (pH 7.4) and kept on ice. Embryos were then homogenised in sterile millipore-filtered PBS (pH 7.4) at a volume-volume ratio of 1 part tissue to 9 parts PBS (pH 7.4). This homogenate was then transferred to an Eppendorf tube using a drawn out siliconised sterile Pasteur pipette and the tube was placed on ice. The homogenate was then used immediately in the bioassay.

5.2.7. The L929-8 TNF bioassay[2]

The L929-8 TNF cytolytic bioassay was used to determine the concentration of TNFα-like activity in day 3 (stage 18) embryos and the percent viability of L929-8 cells in the presence of embryo homogenate with and without neutralising antibodies to mouse TNFα. L929-8 cells are a highly TNFα-sensitive subclone of the murine fibroblastoid cell line L929 (Branch *et al.*, 1991). L929-8 cells were washed and plated at a density of 5×10^4 cells/50μl/well in Iscove's modified Dulbecco's medium (Gibco) containing 10% v/v foetal calf serum (FCS) supplemented with 2μg/ml actinomycin D (Sigma) in 96 well tissue culture flat-bottom plates. After 1 hr of pre-incubation at 37°C, serial dilutions of chick embryo homogenate (50μl) were added to the L929-8 cells. Recombinant murine TNFα (1.2×10^7 U/mg, Genentech) standards were included in each assay. Plates were then incubated for 18

[2] *The L929-8 bioassay was carried out with the help of Peter Lapchak.*

hrs at 37°C and then 50µl of 0.05% (w/v) neutral red (Sigma) in normal saline was added to each well to stain viable cells. Plates were then incubated for a further 2 hrs at 37°C, the media was decanted, the plates were washed in PBS and 100µl of 50% ethanol in 0.05M sodium phosphate was added. The optical density was determined spectrophotometrically (reference wavelength = 650 nm; sample wavelength = 450 nm). Concentrations of proteins with TNFα-like activity could then be calculated from the standard curve. Each experiment was repeated three times, and similar results were obtained in each case. Confirmation of the L929-8 cell lytic activity in the homogenate as TNFα-like was obtained by pre-incubation of homogenate in the presence of 50µl of one of two polyclonal neutralising antibodies to mouse TNFα (Ab1: anti-mouse TNFα, Genzyme; or Ab2: anti-mouse TNFα, Genentech) at a dilution of 1:500. Percentage viabilities of L929-8 cells at particular serial dilutions of embryo homogenate, with and without antibody, were calculated using the following formula:

$$\% \text{ viability} = \frac{OD_{sample} - OD_{100\% \text{ dead}}}{OD_{100\% \text{ viable}} - OD_{100\% \text{ dead}}}$$

Where, OD_{sample} = average optical density from samples of homogenate at a particular dilution; $OD_{100\% \text{ dead}}$ = average optical density of samples at 5pg/ml exogenous TNFα (100% killing of L929-8 cells); $OD_{100\% \text{ viable}}$ = average optical density of triplicate samples at 0.01pg/ml exogenous TNFα (100% survival of L929-8 cells).

5.3. Results

5.3.1. Areas of programmed cell death identified using TUNEL

Using TUNEL, cells undergoing DNA fragmentation during the early development of the chick embryo have been identified. These cells are present in several well characterised regions of cell death including the limb buds, the heart, spinal motoneurons, dorsal root ganglia, and the ventral horns of the neural tube, confirming that TUNEL is a reliable method for the detection of cells undergoing programmed cell death. In addition, other areas of cell

death were identified, including the floor plate, some cells of the myotome, and the mesonephric mesenchyme and Wolffian duct.

i. The limb buds (Day 4; stage 23): Cells undergoing DNA fragmentation were detected in the distal subridge mesenchyme and apical ectodermal ridge (Figs. 5-1a, b) and in the central core of the limb bud (Fig. 5-1a).

ii. The floor plate (Days 3 and 6; stages 18 and 29): A region of cell death was identified in the floor plate of the neural tube. DNA fragmentation, identified by TUNEL, was present in several cells in this region of the neural tube. Dying cells in the floor plate were first identified at the level of the wing buds at stage 18 (Fig. 5-2a). This pattern of cell death was still prevalent in the cells of the floor plate in the tail bud of embryos at stage 29 (Fig. 5-2b).

iii. The nervous system (Day 6; stage 29): Massive cell death in nervous tissue was revealed using TUNEL. As can be seen in Figs. 5-3a and 5-3b, TUNEL-positive cells are present in the ventral aspect of the dorsal root ganglion and in spinal nerves. Under higher magnification (Fig. 5-3b), some of the TUNEL-positive nuclei exhibit the morphology that has been associated with that of apoptosis (condensed chromatin and membrane blebbing). Other nuclei look dense and homogeneous, which is the more commonly described appearance of dying neurons.

5.3.2. Areas exhibiting a positive correlation between TUNEL-positive cells and regions of TNFα-immunoreactivity

In several regions of the trunk, a positive correlation was found between the presence of TUNEL-positive cells and regions of TNFα-immunoreactivity. These regions included the ventral horn of the neural tube, spinal nerves, paravertebral ganglia, mesenchyme of the body wall, the mesonephric mesenchyme and the Wolffian duct, some parts of the myotome, and cranial nerves and ganglia.

i. The Trunk (Day 5; stage 26): Immunocytochemical localisation of TNFα-immunoreactivity using monoclonal anti-mouse TNFα (Fig. 5-4a) revealed an identical pattern of expression to that described previously using a polyclonal antibody (see Chapter 4). Staining for TNFα was evident in the marginal zone of the neural tube and less intensely in the ventral horn of the neural tube, in the ventral nerve, and in the paravertebral ganglia (Fig. 5-4a). When TUNEL was performed on sections adjacent to those stained with the antibodies to TNFα, it became apparent that a positional correlation existed in some tissues between the expression of TNFα-cross-reactive proteins and the presence of fragmented DNA in cells of the same tissues. Dying cells were present in the ventral horn of the neural tube (Fig. 5-4b) and there was some TNFα immunoreactivity in this area (Fig. 5-4a), although this was not as intense as that observed in the marginal zone (Fig. 5-4a), . The ventral nerve was intensely immunoreactive and TUNEL- positive cells were observed in this tissue (compare Figs. 5-4a and 4-4b). The paravertebral ganglia were also highly TNFα-immunoreactive at this stage and a direct correlation was evident between the presence of TNFα-immunoreactivity in this tissue and the presence of dying cells (compare Figs. 5-4a and 5-4b).

ii. The mesonephros (Day 5; stage 26): Cells in the mesonephric mesenchyme and in the walls of the Wolffian duct were found to be undergoing cell death at this stage of development. TNFα-immunoreactivity was also present in these tissues (Figs. 5-5a and 5-5b).

iii. Mesenchyme of the body wall (Day 6; stage 29): It was noted that a particular area of mesenchyme near the gut and adjacent to the body wall had a high number of TUNEL-positive cells, many of which exhibited the classical apoptotic features (Figs. 5-6b and 5-6c). In addition, some cells of the ectoderm appeared to be dying (Fig. 5-6b). In an adjacent section, TNFα- immunoreactivity was present throughout the mesenchyme, but showed foci of particularly intense staining (Fig. 5-6a). Cells of the ectoderm were also stained with these antibodies to TNFα (Fig. 5-6a).

iv. Ectoderm: TNFα-positive cells of the ectoderm of the body wall immediately adjacent to the limb bud are shown in Fig. 5-7a. These cells are intensely TUNEL-positive and their presence correlates with the presence of TNFα-immunoreactivity throughout the ectoderm.

v. The myotome: The myotome stained intensely with monoclonal antibodies to TNFα. When double labelling was used to stain cells of the myotome with antibodies to TNFα (blue) and TUNEL (red), it was noticed that cells in the ventral aspect of the myotome were TUNEL-positive and that this correlated with the presence of TNFα-immunoreactivity in this tissue (Figs. 5-8a and 5-8b).

vi. The heart: Dying cells were observed in the walls of the heart using TUNEL. However, TNFα immunoreactivity in the heart did not appear to correlate wholly with the presence of these cells, although there did appear to be more TUNEL-positive cells (red) in regions of TNFα immunoreactivity (blue; Fig. 5-9).

vii. Cranial nerves and ganglia: When double labelling was carried out on sections through the head (Fig. 5-10), it was observed that cranial nerves and ganglia were immunoreactive for TNFα (blue; see also Chapter 4). These tissues were also found to contain many TUNEL-positive cells (red), but TUNEL-positive cells were also particularly abundant in immediately adjacent tissue (Fig. 5-10).

5.3.3. The L929-8 TNF bioassay[3]

Chick embryo homogenate was incubated with TNFα-sensitive L929-8 cells, both alone and in the presence of two different neutralising antibodies to TNFα (Ab1 and Ab2; see section **5.2.7.**). The optical density of neutral red staining of the cells was used as a measure of the level of L929-8 cell death that had occurred (the lower the optical density the greater the cell killing; Fig. 5-11a). With embryo homogenate, the mean optical density was 0.237 (±

[3] *Raw data is presented for the L929-8 bioassay in Appendix I.*

0.025 estimated SEM, $n = 6$), compared with mean values, obtained using embryo homogenate with antibodies to TNFα, of 0.414 (± 0.008 estimated SEM, $n = 4$; Ab1) and 0.396 (± 0.033 estimated SEM, $n = 4$; Ab2). The value obtained with embryo homogenate alone was significantly different from the value obtained with embryo homogenate with either neutralising antibody to TNFα ($p<0.005$, Ab1 and $p<0.005$ Ab2).

The percent viability of L929-8 cells was calculated from the optical density values presented above and from values obtained in the presence of exogenous TNFα (Fig. 5-11b). Maximum percent viabilities were observed with 0.01 pg/ml TNFα (100%) and with embryo homogenate with neutralising antibodies to TNFα (100% Ab1 and 95.6% Ab2). The minimum percent viability under the present conditions was seen with 5 pg/ml TNFα (3.83%) while embryo homogenate alone gave a value of 57.11%. It was found that the presence of embryo homogenate had the effect of increasing apparent cell viability by several percent in all assays, presumably by promoting L929-8 cell proliferation, therefore the data presented above were normalised to 100% for cells in the presence of both antibody and embryo homogenate to take into account this proliferative effect.

Using a standard curve, of optical density against increasing concentrations of exogenous TNFα (0.01 pg/ml to 5 pg/ml) added to L929-8 cells (see Appendix I-3), it was possible to calculate the mean concentration of bioactive TNFα present in a 1:36 dilution of day 3 (stage 18) embryo homogenate. From this value, the concentration of bio-active TNFα in whole day 3 (stage 18) embryos could be calculated. This was found to be 9.5 (± 5.2 estimated SEM, $n = 6$) pg/ml of undiluted embryo homogenate without antibodies, compared to complete elimination of TNFα-like activity in the presence of neutralising antibodies.

5.4. Discussion

Programmed cell death is a term that is familiar to workers in both the fields of developmental biology and immunology. However, workers in each of the two fields view programmed cell death from different perspectives (Schwartz and Osborne, 1993). Developmental biologists understand the phenomenon as the deletion of cells in a spatially and temporally restricted manner during normal embryonic development as a result of a

physiological stimulus, while immunologists restrict their definition to the death of any cell that requires *de novo* protein expression to bring about its own demise, regardless of the nature of the stimulus (physiological or non-physiological). It has been reported in Chapter 4 that proteins that are cross-reactive with antibodies to TNFα are expressed in some tissues which are known to undergo programmed cell death during early chick embryo development. These observations have been extended in this Chapter using TUNEL to detect cells undergoing the DNA fragmentation indicative of programmed cell death. The present study has shown that a positive correlation exists between TNFα-immunoreactivity and programmed cell death in some locations in the embryo and that TNFα-like cytotoxic activity is present in chick embryo homogenate. These results might suggest that proteins with TNFα-like activity could represent some of the putative "killer proteins" synthesised in tissues undergoing developmental programmed cell death, just as they are in immunological programmed cell death (Hernández-Caselles and Stutman, 1993; discussed below).

The role of TNFα in pathology and inflammation is well documented (Vassalli, 1992), but its physiological functions are not as well understood. Hernández-Caselles and Stutman (1993) have noted that the intrathymic stage of T-cell development is characterised by high rates of cell proliferation and cell death. These authors reasoned that since TNFα is capable of instigating both the death and proliferation of cells, it could have a role in intrathymic T-cell development. This argument is analogous to the proposal presented in Chapters 3 and 4 that since normal embryonic development is similarly characterised by high rates of cell proliferation and death, proteins with TNFα-like activity, when produced locally and at physiological concentrations, could have a role in embryogenesis. Indeed, Hernández-Caselles and Stutman (1993) showed that TNFα influences thymocyte proliferation and apoptosis during intrathymic T-cell development.

It has been suggested that the TUNEL method, and related *in situ* nick-end-labelling techniques, may be *selective* rather than *specific* for cells undergoing programmed cell death; i.e. necrotic cells are also labelled (Ansari *et al.*, 1993). However, as these authors point out, necrotic cells are easily identifiable in tissue sections as large areas of labelled cells rather than scattered TUNEL-positive nuclei such as those that we observed in our experiments. Indeed,

necrotic areas with many TUNEL-positive nuclei were visible in some of the sections, where tissue had obviously been damaged during fixation or processing (not shown). Also, when combined with consideration of the classic morphological criteria described for apoptotic cells, it was apparent that many TUNEL-positive cells also showed this morphology (see Fig. 5-6c). Other cells, though not showing an apoptotic morphology, were TUNEL-positive and were isolated from each other, supporting the contention that TUNEL is able to detect DNA fragmentation in dying cells at early stages, before the morphological criteria for apoptosis are exhibited. Finally, it has been considered that cell division and differentiation is also accompanied by the introduction of nicks into DNA (Ansari *et al.* 1993). However, the TUNEL technique was optimised here such that a very low level of background staining was observed, which was significantly less than the staining observed in TUNEL-positive nuclei, suggesting that any DNA nicks in normal cells were below the level of detection. Therefore, it is considered that the TUNEL method proved to be very effective in detecting cells undergoing programmed cell death *in situ*.

5.4.1. The nervous system

Using TUNEL, the finding that programmed cell death accompanies the normal development of the chick nervous system has been verified. In the lateral motoneurons of the lumbar spinal cord of the chick embryo, it has been noted that 40% of the neuron population degenerates between day 5.5 (stage 28) and day 9 (stage 35) of incubation (Chu-Wang and Oppenheim, 1978). Here, dying cells have been identified in the lateral motor columns (in the ventral horns) of the chick embryo at day 5 (stage 26) and day 6 (stage 29) and this cell death is accompanied by DNA fragmentation, since it is detectable using TUNEL. In addition, dying cells are identified in the paravertebral ganglia and in ventral nerves at this stage. It is confirmed here that TNFα-immunoreactivity of varying intensity can be detected in these areas of the nervous system during these stages of development. This lends support to the proposal put forward in Chapter 4 that TNFα-cross-reactive proteins may be involved in programmed cell death in the nervous system.

It is possible that the TUNEL-positive cells in the ventral nerves are actually Schwann cells that are degenerating following the loss of axons, due to programmed motoneuron cell

death in the ventral horn at this stage. This follows from the suggestion that, in the rat at least, the number of Schwann cells in a developing nerve is matched with the number of axons that are present (Harris and McCaig, 1984). Alternatively, these TUNEL-positive cells could represent the avian equivalent of Schwann cell precursors, which are intermediates in the development of Schwann cells from neural crest cells and have been shown to undergo programmed cell death in the rat embryo (Jessen *et al.*, 1994). Thus, TNFα-like proteins could be one family of "killer proteins" present within the developing nervous system.

Dying cells have also been identified in the dorsal root ganglia, a tissue which has been consistently negative in our studies of TNFα immunoreactivity. This suggests that if TNFα-like proteins are involved in programmed cell death in the nervous system, they may be involved only in certain regions. Furthermore, the identification of cells undergoing DNA fragmentation in the floor plate is a novel finding and suggests that cell death may be involved in the differentiation of this important region of the nervous system. Indeed, Homma *et al.* (1994) have also reported cell death in the floor plate at this time.

In the present study, some dying cells in the nervous system showed classic apoptotic morphological features, while others had nuclei which stained homogeneously. The latter could represent nuclei in the early stages of DNA fragmentation, which have yet to undergo the morphological changes associated with apoptosis. Indeed, in recent work using the TUNEL technique adapted for the scanning electron microscope, it has been revealed that some nuclei, in cells from regions of embryos known to undergo programmed cell death, incorporate high amounts of avidin-gold conjugate even before the morphological changes associated with apoptosis become apparent in these cells (Sanders and Wride, 1995; 1996).

5.4.2. The mesonephros

The presence of TUNEL-positive cells in two different areas of the mesonephros, the mesonephric mesenchyme and the Wolffian duct, was particularly interesting. The development of the mesonephros has been described by Friebová (1975). The observation that programmed cell death is present in the mesonephros is new and it complements recent reports of programmed cell death accompanying the development of the metanephros (Koseki *et al.*, 1992; Coles *et al.*, 1993). It has been proposed that the metanephric mesenchyme is

programmed for apoptosis and that there are two steps to the inductive signal from the ureteric bud to the mesenchyme. The first is the prevention of apoptosis in the mesenchyme while the second is the conversion of the mesenchyme to epithelium, followed by differentiation (Koseki *et al.*, 1992). Similar morphogenetic events may occur in the mesonephros during early chick development and since dying cells have been identified in the mesonephric mesenchyme, this tissue may also have an inherent potential to undergo programmed cell death. The chick mesonephric mesenchyme and the Wolffian duct have also been shown here to have TNFα-immunoreactivity, indicative perhaps of a role for TNFα-like proteins in programmed cell death in these tissues. The significance of dying cells in the mesonephros at these stages remains undetermined, but it is unlikely to be the cause of regression of this tissue, since this is not thought to occur until day 9 (stage 35) of embryonic development (Friebová 1975).

5.4.3. The limb buds

The developing limbs have proven to be an excellent model system in which to study the apparent paradox of cell death during development (reviewed by Hinchliffe, 1981). TUNEL-positive cells were identified in several regions including the opaque zone, within the central core cells, and in both the apical ectodermal ridge and the distal subridge mesenchyme. The presence of programmed cell death in the distal subridge mesenchyme of the chick is a new observation, since previously cell death in this region has only been detected following the removal of the apical ectodermal ridge (Rowe *et al.*, 1982). The apical ectodermal ridge is clearly intact in the studies described here (see Fig. 5-1b), suggesting that the distal subridge mesenchyme is a region of naturally occurring programmed cell death. The presence of TNFα immunoreactivity in the developing non-chondrifying mesenchyme of the limbs was described in Chapter 4, but in the present study a complete correlation between the expression of TNFα-cross-reactive proteins and dying cells identified by TUNEL, in all regions of the limb bud, was not made.

5.4.4. The heart

The heart is another well known site of programmed cell death (Pexieder, 1975). Cell death was identified here in the heart throughout the stages that were examined, but dying cells were particularly evident by stages 26-29. Previously, the presence of TNFα-cross-reactive proteins was shown in Western blots of day 4 (stage 23) heart tissue homogenate (see Chapter 4). TNFα immunoreactivity is shown here to be present in the heart, particularly in the trabeculae and in the developing muscle of the walls of the ventricles. However, the expression of TNFα-cross-reactive proteins in the heart, like the limb, was not restricted to regions where cell death occurred. This does not necessarily preclude a role for TNFα-cross-reactive proteins during programmed cell death in the heart or limb, but rather suggests that they may have additional roles in these tissues, perhaps in extracellular matrix remodelling and as growth and proliferation factors (see Chapter 3).

5.4.5. The L929-8 TNFα Bioassay

The L929-8 cell bioassay was used to detect TNFα-like activity in sterile homogenate from day 3 (stage 18) embryos. The sensitivity of this cell line is highly specific for TNFα and TNFß (for a complete discussion see Branch *et al.* 1991). The embryo homogenate, in the absence of antibodies to TNFα, significantly reduced the viability of the L929-8 cells compared to homogenate in the presence of antibodies to TNFα, implying that the death of L929-8 cells is specifically due to TNFα-like cytotoxic factors present within the embryo homogenate. This result supports the contention that the TNFα-like activity in the embryo homogenate is specifically due to the TNFα-cross-reactive proteins described in Chapter 4. However, at the present time, the exact nature of these TNFα-related proteins remains uncertain, since they have yet to be cloned and sequenced. Furthermore, the mean concentration of TNFα-like activity in embryo homogenate was found to be 9.5 pg/ml, a value that is within the range of physiological (un-stimulated), rather than pathophysiological, levels of TNFα. It is important to emphasise that this is a *mean* value and that the two TNFRs have Kds in the pg/ml range (Rothe *et al.*, 1992). Therefore, in order to have biological effect and assuming that they can interact with the TNFRs (since they do so, at least with the TNFR that mediates cytotoxicity, in

the L929-8 bioassay), TNFα-like proteins must be present in concentrations that exceed the Kds for their receptors in embryonic tissues. From the immunohistochemical data (see Chapter 4 and the present results), it is clear that the distribution of TNFα-cross-reactive proteins in the embryo is not homogeneous, suggesting that these proteins are present in sufficiently high concentrations to bind to TNFRs in tissues in which they are expressed. The results of the bioassay described here support data obtained by Yamasu *et al.* (1989) who showed that mouse foetuses secrete biologically active TNFα in the absence of exogenous stimuli, such as treatment with LPS.

5.4.6. Concluding Comments

Programmed cell death is followed by ingestion of dying cells by macrophages or other phagocytes (Savill *et al.*, 1993). Macrophages are potent producers of TNFα (Vassalli, 1992), and macrophage-like cells, derived from the yolk sac, have been identified in the avian embryo between 2.5 and 4.5 days of incubation (Cuadros *et al.*, 1992; 1993). Furthermore, these macrophage-like cells are present in regions of programmed cell death. Therefore it is possible that at least some of the TNFα, detected by immunohistochemistry in the developing chick, could be produced by macrophages moving into areas of cell death in order to clear dead cells. However, phagocytic macrophages are unlikely to be the only producers of TNFα in developing embryos, since Yamasu *et al.* (1989) have shown that macrophages are not the primary producers of TNFα during mouse development. Therefore, production of TNFα-like proteins may potentially be a cause as well as a consequence of cell death during development.

It may be significant, when suggesting a potential role for TNFα-like proteins in programmed cell death that the p75 low affinity nerve growth factor receptor ($p75^{NGFR}$) constitutively induces neural cell death unless it is bound by NGF (Rabizadeh *et al.*, 1993). This is particularly interesting since $p75^{NGFR}$ is a member of an emerging family of receptors and ligands belonging to the NGF/TNF receptor family (reviewed by Bazan, 1993; see Chapter 2). In the nervous system, loss of support by trophic factors, such as NGF, could result in the death of neurons, due to apoptosis initiated by $p75^{NGFR}$ or perhaps due to the direct action of TNFα-like proteins on their receptors, as is suggested by the present results. Thus, TNFα-like

proteins and their receptors, NGF, trophic factors, and oncoproteins such as bcl-2, which inhibits cell death, could be part of a general cell death system (the "social controls"; Raff, 1992; Raff *et al.*, 1993), which is used to initiate and control cell death during embryonic development. In fact, an involvement for TNFα in the "social controls" of cell survival and cell death has already been suggested for its roles in T-cell production in the thymus (Hernández-Caselles and Stutman, 1993).

In conclusion, this study has shown that chick embryos possess endogenous TNFα-like cytolytic activity at early stages of development, these TNFα-like proteins are produced at physiologically relevant concentrations in the embryo, and they are expressed in some tissues undergoing programmed cell death detected by TUNEL. This lends support to the previous proposal that one potential role for TNFα-like proteins during early embryonic development could be in programmed cell death (see Chapters 3 and 4). Further support for this suggestion comes from studies on gastrulating (stage 5) embryos (Sanders *et al.*, 1996b). In this study, it was revealed that TNFRs, as well as an approximately 17kDa TNFα-cross-reactive protein, are expressed at this stage. Cell death was promoted in cultures of stage 5 embryonic tissue by the addition of TNFα, agonistic antibodies to TNFRs, and neutralising antibodies to TNFα. It was proposed that TNFα may act in a paracrine manner at this stage by activating cell death signalling pathways and by acting indirectly to promote cell death through effects on integrin-mediated cell adhesion (see Chapter 3). It is likely that TNFα-like molecules could have similar effects on embryonic cell death during the stages of development studied in this Chapter. However, there are likely to be many mechanisms of programmed cell death in embryos and it is therefore unlikely that TNFα-like proteins could be involved in *all* examples of programmed cell death. This is evident in the work described in this Chapter, since some regions of cell death detected by TUNEL are not immunoreactive for TNFα; e.g. the dorsal root ganglia. Indeed, it is possible that TNFα-like proteins also have important roles during development in the remodelling of the ECM, the expression of CAMs and integrins, and in cell growth and differentiation (see Chapter 3).

TABLE 5-1
Summary of ODs for L929-8 bioassay.

	Homogenate alone	Ab 1	Ab 2
Mean	0.237	0.414	0.396
Max	0.297	0.434	0.494
Min	0.123	0.396	0.347
n	6	4	4
Est. St. E.	0.025	0.008	0.033
St. Dev.	0.062	0.016	0.066

Figure 5-1. TUNEL labelling of section through the wing bud (Day 4; stage 23). (a). TUNEL-positive cells are present in the central core cells (bracketed) and in the distal subridge mesenchyme (arrowhead). Bar = 100μm. **(b).** Higher magnification of (a) showing details of the TUNEL-positive cells in the apical ectodermal ridge (arrowhead) and distal subridge mesenchyme (arrow). Bar = 50μm.

Figure 5-2. TUNEL labelling of sections through the nerve cord. (a). Day 3 (stage 18). Section through the neural tube (nt) and notochord (n) at the level of the wing buds. TUNEL-positive cells are present in the floor plate of the neural tube (arrow). Bar = 30μm (Fig. 2A, B). **(b).** Day 6 (stage 29). Section through the notochord and neural tube in the tail bud where TUNEL-positive cells persist in the floor plate (arrow).

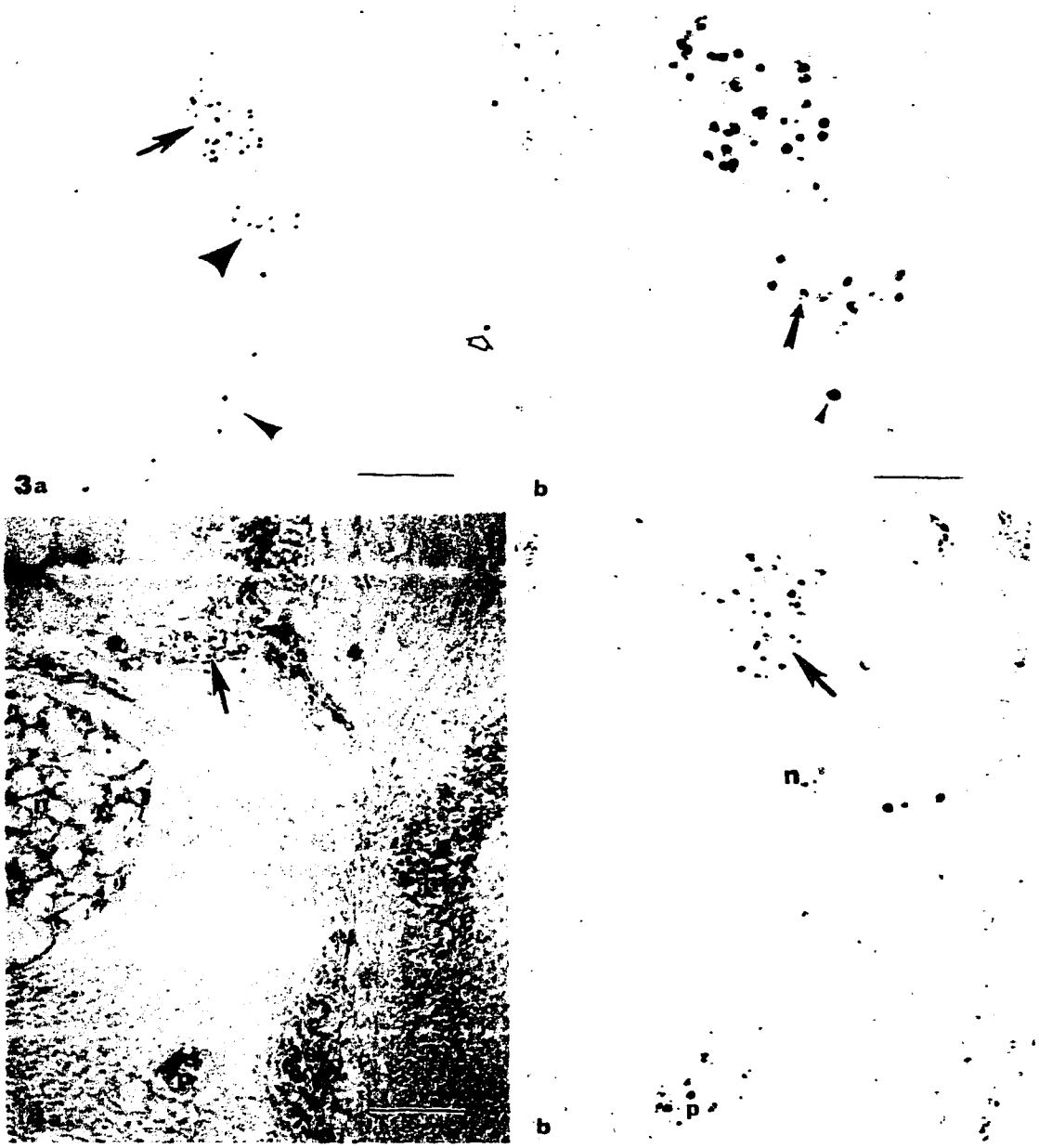

Figure 5-3. TUNEL labelling of section through the trunk at the level of the wing bud (Day 6; stage 29). **(a).** Massive cell death in the ventral aspect of the dorsal root ganglion (arrow), the ventral nerve (large arrowhead), and the spinal nerve (small arrowhead). Note the positive nucleus in the notochord (open arrow). Bar = 100μm. **(b).** Higher magnification of (a). Some TUNEL-positive cells in nervous tissue appear to exhibit the characteristic blebbing morphology of apoptosis (arrow) while others appear to look homogeneous (arrowhead). Bar = 50 μm.

Figure 5-4. Closely adjacent sections through the trunk stained for TUNEL and TNFα (Day 5; stage 26). **(a).** Immunocytochemical localisation of TNFα. Staining is present in the notochord (n), in the marginal zone of the neural tube (arrow), in the myotome (m), in the paravertebral ganglia (p), in the floor plate (f) and in the spinal nerve (s). Bar = 50 μm (Fig. 5-4a, b). **(b).** Section through the trunk in the same region of the same embryo as in (a), but stained using TUNEL. TUNEL-positive cells are present in the ventral horn and marginal zone of the neural tube (arrow), in the ventral nerve root (n) and in the paravertebral ganglion (p). In other sections, labelled nuclei could also be found in the floor plate, notochord, and myotome.

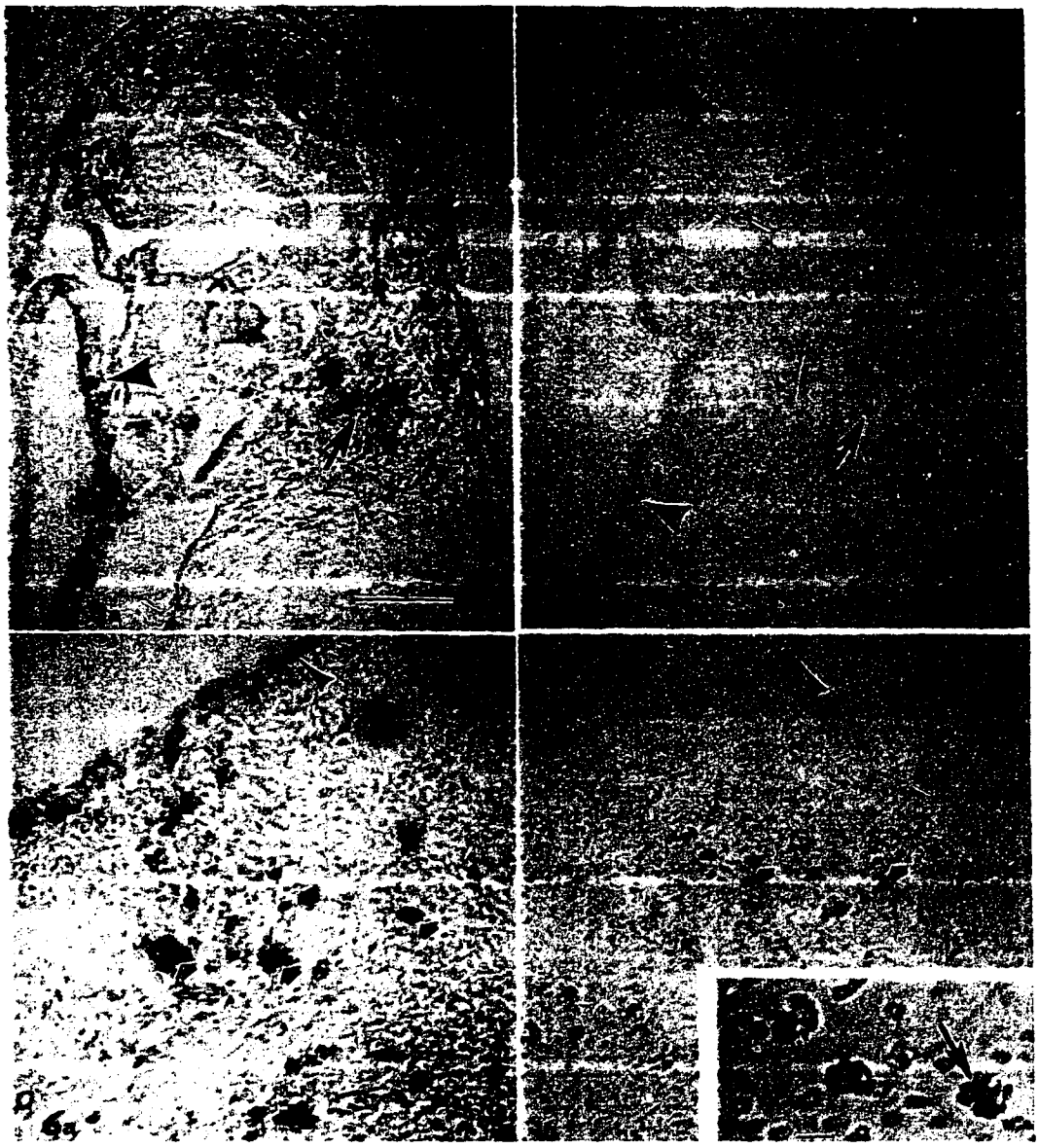

Figure 5-5. Closely adjacent sections through the mesonephros stained for TUNEL and TNFα (Day 5; stage 26). **(a).** Immunocytochemical localisation of TNFα in the mesonephric mesenchyme (arrow) and in the wall of the Wolffian duct (arrowhead). Bar = 50μm (Fig. 5-5a, b and 5-6a, b). **(b).** Section through the mesonephros in the same region of the same embryo as in (a), but stained using TUNEL. Dying cells identified by TUNEL are present in both the mesonephric mesenchyme (arrow) and in the wall of the Wolffian duct (arrowhead).

Figure 5-6. Closely adjacent sections through the body wall adjacent to the gut stained for TUNEL and TNFα (Day 6; stage 29). **(a).** Immunocytochemical localisation of TNFα in the mesenchyme and in particular in several intensely stained foci (arrows). Staining is also present in the ectoderm (arrow head). **(b).** Section through the body wall in the same region of the same embryo as in (A), but stained using TUNEL. TUNEL-positive cells are present in the mesenchyme (arrows) and ectoderm (arrowhead) of the body wall. **(c).** Higher magnification of (b) showing apoptotic cells in the mesenchyme. Cells show the membrane blebbing characteristic of apoptosis (arrow).
Bar = 10μm.

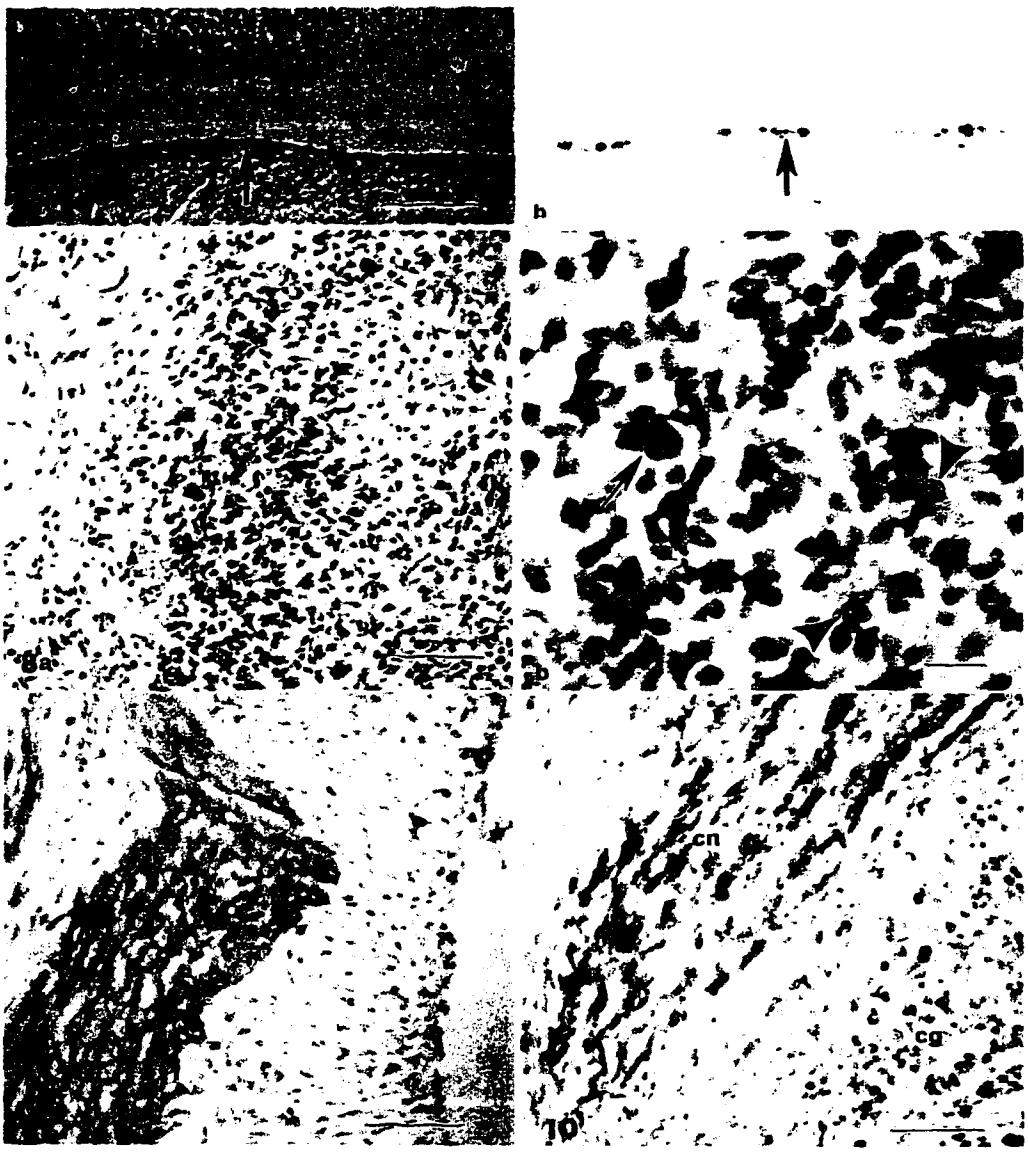

Figure 5-7. Section through the body wall adjacent to the limb bud stained for TUNEL and TNFα (Day 6; stage 29). (a). Immunocytochemical localisation of TNFα in the ectoderm of the body wall (arrow). Bar = 50μm (Fig. 5-7a, b). **(b).** Adjacent section through the body wall in the same region of the same embryo as in (a) showing TUNEL-positive cells in the ectoderm (arrow).

Figure 5-8. TUNEL and TNFα double labelled section through the trunk (Day 6; stage 29). (a). TNFα immunoreactivity in the myotome (dark blue) and apoptotic cells (red). Bar = 50μm. **(b).** Day 6 (stage 29). Higher magnification of (a). One cell (arrow) shows the classic membrane blebbing of apoptosis, is TUNEL-positive (red), and also appears to be TNFα-immunoreactive (dark blue). Other TUNEL-positive cells appear to be stained dark blue for TNFα at their peripheries (arrowheads). Bar = 10μm.

Figure 5-9. TUNEL and TNFα Double labelled section through the heart (Day 6; Stage 29). TNFα immunoreactivity is seen primarily in the developing cardiac muscle of the ventricle wall (dark blue) while TUNEL-positive cells (red) are also seen in this tissue. Bar = 50μm.

Figure 5-10. TUNEL and TNFα double labelled section through the head (Day 5; stage 26). (a). The cranial nerve (cn) is immunoreactive for TNFα (dark blue) and contains TUNEL-positive cells (red), while the adjacent cranial ganglion (cg) also contains many TUNEL-positive cells (red). Bar = 50μm.

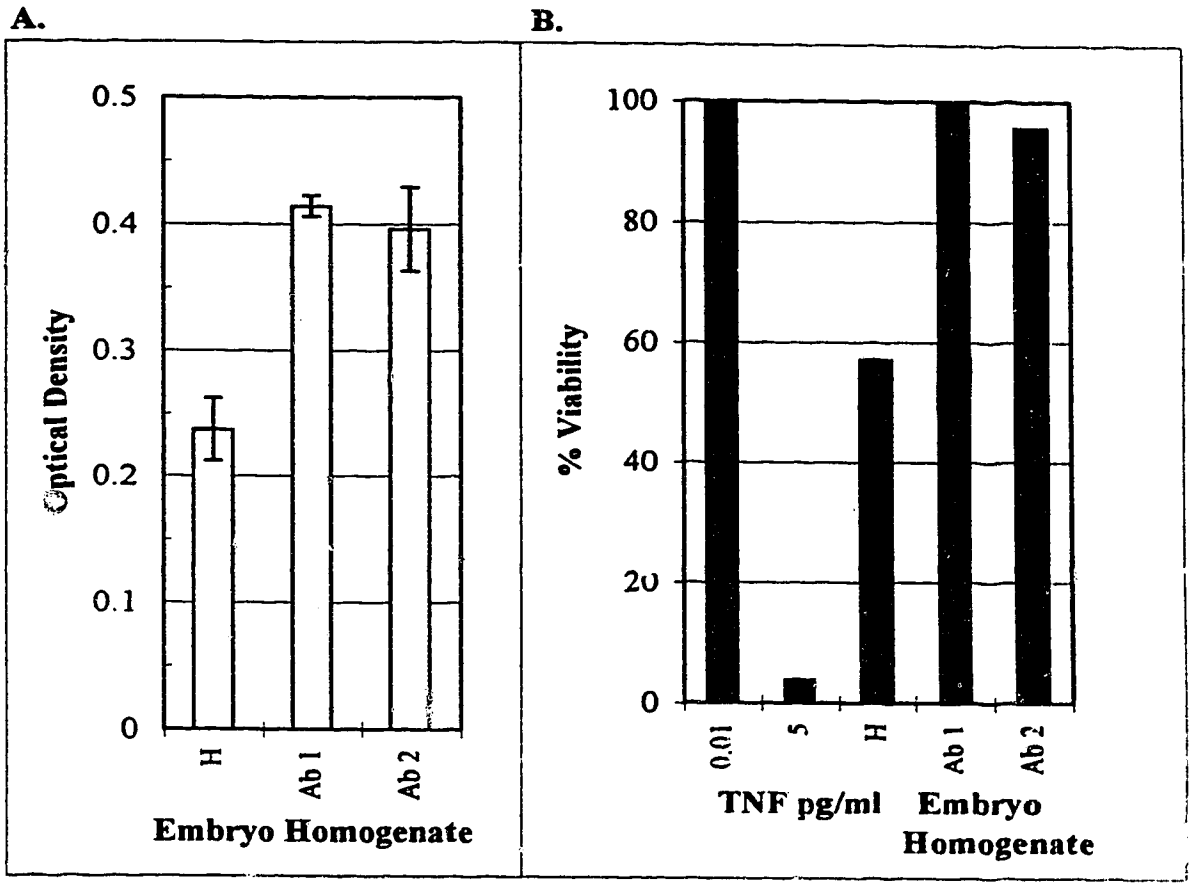

Figure 5-11. The L929-8 TNFα bioassay. (A). The effect of day 3 (stage 18) chick embryo homogenate at a final dilution of 1:36 on the optical density of L929-8 cells alone (H) or in the presence of neutralising antibodies to TNFα (Ab1, Genzyme and Ab2, Genentech). The optical density is significantly reduced (implying increased cell death) in the presence of embryo homogenate compared to that with embryo homogenate in the presence of TNFα specific neutralising antibodies ($p<0.005$, Ab1 and $p<0.005$, Ab2). **(B).** The percent viability of L929-8 cells challenged with exogenous TNFα at concentrations of 0.01 pg/ml and 5 pg/ml or with embryo homogenate at a final dilution of 1:36 alone (H) or embryo homogenate in the presence of neutralising antibodies to TNFα (Ab1, Genzyme and Ab2, Genentech). Bars = mean ± estimated SEM.

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PART III: THE DEVELOPING LENS: FUNCTIONAL STUDIES

CHAPTER 6

THE DEVELOPING LENS: A MODEL SYSTEM FOR THE STUDY OF CELLULAR AND MOLECULAR EVENTS IN EMBRYOGENESIS[1]

6.1. Introduction

The developing lens is an excellent model system for the study of many fundamental morphogenetic processes occurring during embryonic development (Bloemendal, 1977; Piatigorsky, 1981). Examples of these processes include: the induction of the presumptive lens ectoderm, as a result of interactions with the optic vesicle; invagination of the ectoderm and formation of the lens vesicle, accompanied by programmed cell death and cell elongation; tightly regulated rates of lens cell proliferation; deposition of distinct families of crystallin proteins, which are the structural components of the lens; the formation of membrane specialisations, including integrins and CAMs for cell-matrix and cell-cell adhesion and gap junctions for cell communication; remodelling of the cytoskeletal structures; and the loss of DNA and the degradation of nuclei and organelles in lens fibres.

Furthermore, studies that add to the understanding of lens development also contribute to a greater understanding of ocular diseases, such as cataract, since these diseases can often be better understood within a developmental context (Coulombre, 1979). In addition, since the development of the lens is an ongoing process that continues beyond embryogenesis, it is also a good model for the investigation of the ageing process (Piatigorsky, 1981).

In this thesis, it has been noted that the developing lens of the chick embryo expresses proteins that are cross-reactive with antibodies to TNFα (see Chapter 4). Therefore, in Chapter 7, the developing avian lens is used as a model system to investigate potential roles for TNFα in lens development. Specifically, the role of TNFα in the degeneration of lens fibre cell nuclei is investigated. In order to put this work in context and to provide an understanding of lens development and the types of studies that have been performed, using the lens as a model

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system, the more recent literature pertaining to lens development is reviewed in this Chapter in relation to some of the older work.

6.2. Lens induction and early embryology

Lens morphogenesis was instrumental in the development of the concept of embryonic induction; i.e. that one tissue (the inducer) can produce factor(s) that diffuse towards, and act upon, another tissue (the responding tissue), which is in close proximity to the inducer, in order to change the developmental fate of the responding tissue. In this way, the orderly arrangement of different types of tissue in the embryo can be explained in terms of the production and reception of signals during sequential tissue interactions. With specific regard to lens induction, the classic proposal was that developed following the experiments of Spemann (1901) and Lewis (1904; cited by Grainger, 1992). This proposal was that the optic vesicle produces factor(s) that are necessary, and sufficient, for the induction of lens tissue in embryonic ectoderm of any type.

In recent years, however, this classical interpretation of lens induction has been reinvestigated and it has been shown that the process of lens induction is more complicated than originally thought (Grainger, 1992; Grainger *et al.*, 1992; Saha *et al.*, 1992).

Grainger *et al.* (1988) reconsidered the sequence of events occurring during lens induction in amphibians and argued that the optic vesicle is not generally sufficient to induce lens in ectoderm. Instead, they proposed a model in which lens induction occurs by a multi-step process in which its essential phases occur before contact of the ectoderm with the optic vesicle (Fig. 6-1). For example, it was proposed that tissue interactions as far back in development as gastrulation are required to confer a lens-forming bias on a large area of ectoderm, which is then acted upon by the lens vesicle to induce lens formation in a defined area (Saha *et al.*, 1989). More specifically, this lens-forming bias may be conferred by presumptive cardiac mesoderm in contact with the epiblast (Jorquera *et al.*, 1989).

Lens induction in the chick embryo begins at about 35 hours of incubation and results in elongation of the cells in the presumptive lens placode between 44 and 50 hours and invagination of the placode between 50 and 55 hours, while δ-crystallin synthesis begins in the presumptive lens placode at about the same time that cell elongation begins (Shinohara and

Piatigorsky, 1976). This programme of events is comparable to that occurring in the mouse, where the only difference is that α-crystallin is the main protein synthesised in the responding tissue (Zwaan, 1983). It has been proposed that the role of the optic cup in lens induction is to provide a high local concentration of a putative growth factor, bound to its ECM, which could be the same as the factor that induces secondary lens fibre induction later in development (Head *et al.*, 1992; see below).

The early morphogenesis of the eye, including the lens, is accompanied by morphogenetic movements including invagination, fusion and separation (Schook, 1978), which are brought about by the interplay of mitosis, cell differentiation, and cell migration (Schook, 1980) as well as programmed cell death (see below). Moreover, a mathematical formulation of lens placode invagination has been developed, which helps to explain how growth pressure alone could be the driving force for tissue folding (Hendrix *et al.*, 1993).

The orderly sequence of events, resulting in lens development, following lens induction, is summarised in Fig. 6-2. Specifically following induction, formation of the lens vesicle and elongation of the primary lens fibres, the lens develops further by processes of cell proliferation and differentiation until it is composed of two cell types: the epithelial cells at the anterior and the secondary fibre cells at the posterior. The lens epithelial cells can be divided into two further cell populations, based on rates of DNA synthesis (Persons and Modak, 1970) The central epithelial cells gradually enter a stationary phase in which their DNA synthetic activity is minimal, while cells in the peripheral epithelium and annular pad continue to synthesise DNA and progressively enter the terminal differentiative programme associated with secondary lens fibre cell formation, which includes cell elongation, crystallin synthesis, a reduction in proliferation rate, and the breakdown of organelles and nuclei (see Piatigorsky (1981) for more details).

Furthermore, it has become apparent that rates of lens epithelial cell proliferation and differentiation into secondary lens fibres are influenced by a number of polypeptide growth factors and cytokines (see below).

6.3. Lens cell culture techniques

One major factor that has facilitated the use of the lens as a model system for the study of many important developmental processes has been the introduction of lens cell culture techniques.

Successful efforts were made to cultivate primary explants of the lens epithelium of chick embryos *in vitro* by Kirby (1927) and Kirby *et al.* (1929), who noted that under certain conditions, influenced by the composition of the incubating medium, lens epithelial cells lived, migrated, and multiplied.

However, the first reports of differentiation of lens epithelial explants *in vitro* did not appear until the 1960's. Philpott and Coulombre (1965) showed that foetal calf serum (FCS) induces transplanted lens epithelial explants to exhibit the histological appearance of early lens fibres and, when incubated with various tissues from the ocular environment, lens epithelial explants exhibit varying degrees of differentiation (Philpott and Coulombre, 1968). Piatigorsky and his colleagues subsequently developed this technique further and showed that the differentiation of lens epithelial explants into fibre-like cells resembled, in many respects, the differentiation of lens fibres occurring *in vivo*. For example, differentiation in culture was accompanied by cell elongation, crystallin upregulation, organelle breakdown, and DNA fragmentation (Piatigorsky *et al.*, 1972a;b; Piatigorsky *et al.*, 1973; Beebe and Feagans, 1981). Similar explant techniques have also been described for rat lens epithelium, which undergoes similar morphological changes in culture (Campbell and McAvoy, 1984; McAvoy and Richardson, 1986).

Techniques were also developed, which depended on the ability of dissociated chicken lens epithelial cells to differentiate in culture into spherical clumps of lens fibre-like cells that have been termed "lentoids" (Okada *et al.*, 1971; 1973) and which exhibit many of the properties of lens fibre cells differentiating *in vivo*, including the deposition of crystallins (Sawada *et al.*, 1992). However, an extended period in culture is required before lentoids appear, using this method, and even then differentiation is not extensive. Subsequently, a similar technique was developed by Menko *et al.* (1984) that allowed development of extensive areas of lentoids, in cultures of dissociated chick embryo lens epithelial cells, after only a few days. Furthermore, lentoid morphogenesis, in culture, paralleled the differentiation of lens

fibres occurring *in vivo* and it is a modification of this technique that is described in more detail in Chapter 7 in relation to studies investigating the effects of TNFα on avian lens morphogenesis *in vitro*.

Thus, lens cell culture techniques have been developed that are valuable in studies investigating the differentiation of lens epithelial cells into fibre cells.

6.4. Growth factors and cytokines

A large number of recent studies have focused on the involvement of polypeptide growth factors and cytokines in lens differentiation. These factors include FGFs, insulin and IGFs, TGFs, PDGFs, EGFs and several cytokines, including MIF, IFN-γ, and TNFα. Recent evidence for the presence and effects of these factors and their receptors in lens development is reviewed below. Further information may be found in McAvoy and Chamberlain (1990) and Tripathi *et al.* (1991a).

6.4.1. Fibroblast growth factor

FGF plays a major role in lens epithelial cell migration and proliferation and also in lens fibre differentiation and growth (McAvoy *et al.*, 1991). When cultured with medium conditioned by neural retina cells, transplanted lens epithelium from newborn rats undergoes morphological changes characteristic of fibre differentiation and this is accompanied by up-regulated crystallin synthesis (Walton and McAvoy, 1984; Campbell and McAvoy, 1986). However, the nature of the factor(s) promoting this differentiation-promoting remained elusive until Chamberlain and McAvoy (1987;1989) showed that FGF was the factor from the retina responsible for lens fibre differentiation. The effects of FGF were dose-dependent with bFGF exerting half-maximal response at 55ng/ml, while aFGF required 290ng/ml for half-maximal response (Chamberlain and McAvoy, 1989). FGF also stimulated cell migration and proliferation in these experiments. The nature of the response was dependent upon the concentration of FGF to which the explants were exposed (McAvoy and Chamberlain, 1989). At the lowest concentrations of FGF (150pg/ml), the cells responded by proliferating, while at higher concentrations (3ng/ml) cell migration was stimulated, and at the highest concentrations (40ng/ml), ß-crystallin synthesis and fibre differentiation were stimulated. An antibody to

bFGF inhibited all responses by 90-93%, thus proving the specific ability of FGF to induce these responses. It has been suggested that the lower concentrations of FGF may act via cell surface receptors to transduce intracellular signals, thereby eliciting cell migration and proliferation, while higher concentrations of FGF may result in translocation of FGF to the nucleus where it might directly stimulate lens fibre differentiation (Peek *et al.*, 1992).

It has been demonstrated that only the vitreous humour, located at the posterior of the lens, can initiate fibre differentiation and ECM accumulation in lens epithelial explants (Lovicu *et al.*, 1995). Interestingly, there appear to be concentration gradients of FGF within the eye (see McAvoy *et al.* (1991) for discussion) and vitreous humour has a substantially higher concentration of FGF than does aqueous humour (Shulz *et al.*, 1993), while bFGF localises to the pigmented and neural retina as well as to the lenses of chick embryos at ED 5-20 of development (Consigli *et al.*, 1993). Similarly, during rat EDs 10-18, both aFGF and bFGF have been localised in the lens and retina (de Iongh and McAvoy, 1993). Furthermore, the expression patterns of two FGF receptor genes (FGFR1 and FGFR2) have been analysed in the developing chicken eye (Ohuchi *et al.*, 1994). FGFR1 is expressed in ocular tissues, including the prospective lens and neural retina, suggesting that it may be the FGF receptor sub-type primarily involved in lens development in the chick.

The observations on spatio-temporal expression patterns, of FGF and its receptors in the developing lens, are consistent with the proposal by McAvoy and Chamberlain, (1989; 1990) and McAvoy *et al.* (1991) that FGF could determine spatial patterns of lens cell proliferation, migration and differentiation by both autocrine and paracrine mechanisms. Thus, FGF can be considered a lens morphogen. Since different levels of FGF stimulation bring about different lens cell responses, the differential distribution of FGF, in different compartments of the lens, or ocular media, could elicit lens polarity and affect growth patterns. This hypothesis has very recently been strengthened by the observations of Robinson *et al.* (1995a). These authors created transgenic mice, over-expressing FGF-1 (aFGF) under the influence of the αA-crystallin promoter. When conjugated to a secretory signal sequence, extracellular expression of the transgene was obtained. Mice expressing secreted FGF-1 exhibited lens abnormalities, particularly the elongation of anterior epithelial cells. These results demonstrate that alteration of the spatio-temporal expression pattern of FGF-1, in the

developing lens, has profound effects on lens development and they highlight the importance of tightly regulated spatio-temporal control of FGF expression in the developing ocular environment. Also, soluble forms of the high-affinity FGF receptor have been identified in human vitreous fluid (Hanneken *et al.*, 1995), suggesting that soluble FGF receptors may also be involved in the regulation of FGF-specific effects during lens development

Lovicu and McAvoy (1992) and Richardson *et al.* (1992) showed that the effects of FGF on lens fibre differentiation may also extend into adulthood and throughout life. In this study, even though there was an age-related decrease in responsiveness to FGF in both peripheral and central lens epithelial explants (as determined by ultrastructural analysis of characteristic morphological changes), peripheral explants from the oldest rats were still able to respond to FGF by up-regulating ß-crystallin synthesis.

Peek *et al.* (1992) showed that, under the influence of FGF, the expression of α-, ß-, and τ- crystallin genes is sequentially activated in rat lens epithelial cell explants. However, once differentiation is complete (days 10-11), all crystallin mRNAs disappear and a new mRNA, aldose reductase, is synthesised and this also occurs under the influence of bFGF

It is important to realise that FGF is not alone in its actions on lens development (as will be seen below) and the particular profile of growth factors present at a given time will influence differentiation in specific ways (Peek *et al.*, 1992). For example, IGF-1 and/or insulin may also have important synergistic roles with FGF in the induction of rat lens cell differentiation (Chamberlain *et al.* 1991; Richardson *et al.*, 1993) and there may be other interactions with factors such as TGFß (Liu *et al.*, 1994).

Finally, FGF may also have a role in cell survival in the lens. Ishizaki *et al.* (1993) showed that, in serum free culture, lens epithelial cells were dependent upon each other for their survival, but the nature of the survival factor(s) remained undetermined. Renaud *et al.* (1994) subsequently demonstrated that the addition of anti-sense oligonucleotides that are specific for aFGF, to bovine lens epithelial cells, resulted in the death of these cells, due to the suppression of endogenous aFGF production. This result suggests that, unlike exogenous aFGF, which has a role in the regulation of cell proliferation and differentiation, endogenous aFGF production appears to be involved in interfering with the actions of genes involved in cell death in the lens epithelium. Furthermore, in transgenic mice expressing a truncated, dominant

negative FGF receptor, several eye defects were noted, including cataracts, severe micropthalmia, reduction in the numbers of lens epithelial cells, reduction of lens fibre elongation, and apoptosis of lens fibre cells, while the pattern of differentiation-specific crystallin expression was normal (Chow *et al.*, 1995; Robinson *et al.*, 1995b). Thus, FGF would appear to be not only a differentiation-promoting factor, but a cell survival factor in the lens.

6.4.2. Insulin and insulin-like growth factor

The effects of insulin and IGF have been studied primarily in the chicken embryo lens, which has proven to be an extremely useful model system for analysis of the effects of IGF during development (de Pablo *et al.*, 1993). Beebe *et al.* (1980) first identified a lens differentiating-promoting activity in chicken embryo vitreous humour, which they called "lentropin". However, the exact nature of this factor remained undetermined until Beebe *et al.* (1987) showed that lentropin was functionally related to IGF-1. Furthermore, lens epithelial cells themselves express functionally active IGF-1 receptors and insulin receptors (Bassas *et al.*, 1987; Alemany *et al.*, 1990). Using radio-iodinated IGF and insulin, it was shown that changes in the binding of IGF and insulin correlated with changes in the growth rate and differentiation state of the cells (Bassas *et al.*, 1987). IGF-1-binding capability was related to growth rate, while insulin-binding capability was correlated with the differentiation state of the cells. The same group (Alemany *et al.*, 1989) also revealed that insulin and IGF-1 were capable of up-regulating chicken δ-crystallin (the major crystallin of chicken lens fibres) gene expression in the developing lens.

The nature of the signal transduction mechanisms of IGF-1 receptors and insulin receptors has also been investigated (Jacobs *et al.*, 1992). It was shown that activation of tyrosine kinase, in response to either hormone, was greater in epithelial cells than fibre cells and also that the insulin receptor was able to enhance tyrosine kinase activity compared to IGF-1, suggesting some subtle, but important, differences between insulin and IGF-1 signalling in the lens.

As was the case with FGF, it was shown that lens epithelial cells are themselves capable of expressing IGF-1, thus adding an autocrine component to the actions of IGF-1 in

lens development in the chick (Caldés *et al.*, 1991). These authors also suggested that the effects of IGF-1 might be similar to those proposed for FGF (see above) in that concentration gradients may be set up by locally produced IGF-1 from the lens epithelial cells and by the IGF-1 in the vitreous humour. Indeed, a recent study emphasises the effects that IGF-1 can potentially have on the migration, proliferation, and differentiation of lens cells (Palmade *et al.*, 1994). Bovine lens epithelial cells in culture were shown to express IGF-1-binding sites and to synthesise and release IGF-1. When cells were incubated with IGF-1, there was a concomitant increase in the expression of fibronectin receptor sites. Thus, IGF-1 may be having some of its effects on lens cell differentiation through an indirect effect on integrins and perhaps CAMs (see below).

Finally, soluble IGF receptors are present in developing chick ocular tissues (Schoen *et al.*, 1995), and in vitreous and aqueous humour, and their levels are regulated by a latent cysteine proteinase activity (Moshyedi *et al.*, 1995); thus, providing a means of regulating the functional activities of IGFs in ocular fluids.

6.4.3. Platelet-derived growth factor

The rat lens undergoes massive growth during the early postnatal period, increasing in weight by a factor of 23 in 26 days (Brewitt and Clark, 1988; 1990). Using an *in vitro* system, these workers have demonstrated that the growth rate per day oscillated between 0 and 87% and that this growth was dependent upon pulsatile delivery of PDGF to the lens. Similarly, pulsatile delivery of PDGF was also required to maintain lens transparency, while continuos delivery of PDGF had no such effect on growth or the maintenance of transparency.

Knorr *et al.* (1993a) were the first to demonstrate that both PDGF receptors (α and ß) are expressed on cultured bovine lens epithelial cells. Receptor binding studies were performed using radio-iodinated PDGF isoforms and it was shown that binding sites for the PDGF-BB isoform exceeded those for the PDGF-AB isoform, which in turn exceeded those for the PDGF-AA isoform. How might PDGF effects be mediated in the lens? In another study (Knorr *et al.*, 1993b), it was demonstrated that stimulation of bovine lens epithelial cells with PDGF isoforms did not result in effects on cell proliferation, but that PDGF-AB and -BB addition resulted in a dose-dependent rise in intracellular free Ca^{2+}, which is an important

second messenger. Addition of PDGF-AA, however, did not produce a rise in Ca^{2+} levels. Thus, at least some effects of PDGF in lens epithelial cells may be mediated by alteration of intracellular Ca^{2+} homeostasis.

The PDGF receptor-α subunit, which is required for the binding of the PDGF-A chain, is expressed through a wide range of stages of mouse embryogenesis in mesoderm- and ectoderm-derived tissues, including the lens (Schatteman *et al.*, 1992). The murine *patch* mutation is due to a deletion in the PDGF receptor-α subunit and it results in a range of anomalies, including disturbances in lens development (Morrison-Graham *et al.*, 1992; Orr-Urtreger *et al.*, 1992).

Potts *et al.* (1993) identified the chicken homologue of the human PDGF α receptor in the chicken lens using the polymerase chain reaction (PCR). In a subsequent study, the same group (Potts *et al.*, 1994) carried out an investigation into the expression and function of PDGF receptors in the developing chick lens. PDGF α receptor was detected in the chicken lens, while PDGF ß receptor could not be detected. Immunoreactivity for PDGF α receptor was detected throughout the lens epithelium at ED 6 and at later stages in the peripheral lens epithelium and annular pad regions. In culture, the addition of exogenous PDGF enhanced proliferation in lens epithelial cells. The authors speculated that PDGF may have a role in the control of lens growth during chick lens development.

6.4.4. Transforming growth factor ß

A number of studies have identified TGFß in the ocular environment. TGFß has been identified both in the aqueous and the vitreous humour (Connor *et al.*, 1989; Granstein *et al.*, 1990; Jampel *et al.*, 1990; Cousins *et al.*, 1991), while three different isoforms of TGFß have been identified in mouse embryo lens fibres (Pelton *et al.*, 1991). Finally, Potts and Beebe (1993) have described expression of TGFß family members during chick lens development.

Kurosaka and Nagamoto (1994) demonstrated that TGFß-2 inhibits lens epithelial cell proliferation in a dose-dependent manner. Human vitreous humour stimulated the proliferation of these cells and this effect could be promoted further by the addition of anti-TGFß-2

antibodies to the vitreous. Thus, TGFß-2 may be considered to be a negative regulator of lens cell proliferation.

Potts *et al.* (1995) have shown that expression of TGFβ2 and TGFβ3, but not TGFβ1 and TGFβ4, coincides with the presence of mitochondria in lens fibre cells and this mitochondrial expression of TGFβs disappears concomitant with the disappearance of lens fibre cell mitochondria in these cells (see below). The possible role of TGFß in rat lens development and growth was investigated recently by Liu *et al.* (1994). It was suggested that TGFß may have a role in normal processes in the lens, such as extracellular matrix remodelling and lens capsule formation. Furthermore, TGFß induced morphological changes in lens epithelial cell explants distinguishable from those elicited by FGF. These changes were characteristic of many of those that occur during the formation of subcapsular cataracts and they included ECM accumulation, capsular wrinkling, cell death by apoptosis and the formation of spindle shaped cells. Furthermore, it has also been shown that TGFß is capable of inducing the accumulation of α-smooth muscle actin, which is a marker for subcellular cataracts, in lens cells (Hales *et al.*, 1994). Thus, studies that identify factors, such as TGFß, that are involved in normal lens differentiation and studies investigating the ways in which these factors interact with each other, may also provide insights into some forms of cataract.

6.4.5. Transforming growth factor α and epidermal growth factor

Since TGFα belongs to the EGF family (Hommel *et al.*, 1992) and it interacts with and activates EGF receptors (Reynolds *et al.*, 1981), these factors will be discussed together in this section.

TGFα is expressed in rabbit and primate lens epithelial cells and it exerts dose-dependent effects on both lens epithelial cell proliferation and on cellular morphology (Wickström and Madsen, 1993). In addition to a normal role in the control of lens growth, it was suggested that the release of TGFα following trauma, such as cataract surgery, may contribute to lens epithelial cell growth. Using the transgenic mouse technique, Decsi *et al.* (1994) and Reneker *et al.* (1995) have shown that overexpression of TGFα, under the influence of the αA-crystallin promoter, results in pathologies of the eye, including the lens.

The results, thus suggest that alteration of normal TGFα expression disrupts the co-ordination of lens development normally involving endogenous lens TGFα production; for example, on perioptic mesenchyme migration (Reneker *et al.*, 1995).

Hongo *et al.* (1993) studied the distribution of EGF receptors in rabbit lens epithelial cells and the effect of EGF on lens epithelial cell growth. It was demonstrated that both low and high affinity EGF receptors are expressed on lens epithelial cells and that EGF enhanced cell growth in a dose-dependent manner, suggesting a role for EGF in lens development. These results are of interest in light of the study of Tripathi *et al.* (1991b), which showed that the quantity of endogenous EGF in cataractous lenses correlated with the clinical stage of the cataract. Furthermore, these authors pointed out that the expression of EGF also correlated with the mitotic activity of the proliferative zone of the lens.

6.4.6. Macrophage-migration inhibitory factor

MIF has recently been recognised as an important pro-inflammatory macrophage cytokine with an important role in endotoxic shock (Bucala, 1994). Interestingly, Wistow *et al.* (1993) showed that MIF is expressed in the developing chick lens and that this expression strongly correlates with differentiation.

6.4.7. Interferon-γ

IFN-γ has not yet been shown to be one of the endogenous factors produced by lens cells, but it has been over-expressed in transgenic mice lenses under the influence of the αA-crystallin promoter (Egwuagu *et al.* 1994). Lens differentiation was grossly disrupted and the authors speculated that these effects were a result of a disruption of the regulation of cytokine responsive transcriptional factors that are important in lens differentiation.

6.4.8. Tumour necrosis factor α

TNFα, like IFNγ and MIF, is also an important immunomodulatory and pro-inflammatory cytokine, which may have a number of roles in embryonic development (see Chapters 2 and 3). Proteins that are cross-reactive for TNFα are expressed in the chick

embryo lens soon after the differentiation of secondary lens fibres begins at four days of incubation (see Chapter 4). Since TNFα is capable of instigating DNA fragmentation and apoptosis in sensitive cells and TNFα immunoreactivity is present in some tissues undergoing programmed cell death during development (see Chapter 5), one role for TNFα in the lens could be the degradation of lens fibre DNA and nuclei during lens fibre differentiation.

6.4.9. Final comments

It is likely that other factors, both well known and yet to be discovered, will provide new insights into the mechanisms of lens development. For example, Hyatt and Beebe (1993) have identified a factor in chick embryo serum, which contributes to the regulation of lens growth and polarity by ensuring that lens epithelial cells divide, but do not differentiate into lens fibres. The nature of this factor remains unknown, but its identification has allowed an update, by these authors, of the proposal by McAvoy and Chamberlain (1989) that variations in FGF concentration may influence the response of lens epithelial cells (as discussed above). Hyatt and Beebe suggest that the situation may, in fact, be more complex and that the presence of inhibitory and stimulatory factors, which are differentially dispersed in the anterior chamber and vitreous body of the eye, may result in the creation of reciprocal gradients that influence lens polarity and morphogenesis.

Also, growth factors have many of their effects on proliferation and differentiation through the activation of proto-oncogenes and, in the embryonic chicken lens, contrasting patterns of c-myc and N-myc protein expression have been observed (Harris *et al.*, 1992). It was demonstrated that c-myc protein is associated with cell proliferation in the lens epithelium, while N-myc expression increased markedly with the onset of lens epithelial cell elongation and terminal fibre differentiation. Since N-myc dimerises with c-myc, it was suggested that formation of c-myc-N-myc heterodimers could contribute towards the reduction in cell proliferation observed in the lens fibres. In transgenic mice overexpressing c-myc or the related molecule L-myc, in their lenses, under the influence of the αA-crystallin promoter, L-myc overexpression resulted in impaired differentiation of the lens, while overexpression of c-myc resulted in deregulated cell cycle progression (Morgenbesser *et al.*, 1995).

Furthermore, it is apparent that cytokines such as MIF, IFN-γ and also TNFα (see Chapter 4), which have been shown to be expressed in the lens during differentiation, may have roles beyond those that they have in the immune system as differentiation factors and/or differentiation factors during development (Wistow *et al.*, 1993; see Chapter 5). Furthermore, the presence of inflammatory cytokines in the lens may also have implications for ocular disease. For example, release of inflammatory cytokines from damaged lenses may contribute to ocular inflammation.

The identification and further study of the functions of the polypeptide growth factors and cytokines described above, as well as others that have yet to be identified in the lens and ocular environment and the effects of the proto-oncogenes they activate, will provide insights into the fundamental process of lens development and may also provide a greater understanding of diseases such as cataract and ocular inflammation in which these factors may have a role. The use of transgenic methods, like those described above that utilise the αA-crystallin promoter to over-express growth factors, cytokines or inhibitors of their expression in the lens, may be particularly useful in this regard.

6.5. Gap junctions and cell-cell communication

In the lens, the formation of gap junctions is extensive. Gap junctions are clusters of cell-cell channels, which connect the cytoplasm of adjacent cells via hydrophilic channels formed by polypeptide subunits called connexins (Kistler *et al.*, 1995). Several connexins, present during lens development, have been characterised; for example, in bovine (Konig and Zampighi, 1995) and avian lenses (Berthoud *et al.*, 1994).

Making use of their lens culture system (Menko *et al.*, 1984; see above), Menko *et al.* (1987) characterised the junctions formed between the cells of lentoids. It was concluded that during differentiation *in vitro* extensive lens fibre junctions and functional cell-cell channels form between cells in lentoids. It was also proposed, by these authors, that proteins, such as the major intrinsic protein (MIP or MP28), which contribute towards the formation of lentoid cell junctions, may have roles in channel formation and intercellular communication vital to the maintenance of the differentiative state in lens fibres. Subsequently, it was demonstrated that both gap junction formation and lentoid differentiation could be inhibited by the addition of the

v-src gene, from the Rous sarcoma virus, to lens epithelial cell cultures (Menko and Boettiger, 1983) despite the fact that MP28 was synthesised at normal levels. Since lens junctions are involved in intercellular communication (Peracchia *et al.*, 1985), the failure of differentiation brought about by *v-src* could be a result of inhibition of intercellular communication pathways involved in the events of differentiation.

Gap junction formation occurs extensively in the mouse lens during development (Evans *et al.*, 1993) and there are changes in connexin expression during chick lens development (Jiang *et al.*, 1995). The sequential changes in gap junction abundance and connexin subtype may reflect distinct functional phases of cellular communication during development of the lens. For example, changes in lens connexin expression, during development in the mouse, have been shown to lead to increases in gap junctional voltage dependence and conductance (Donaldson *et al.*, 1995). Furthermore, lens cell junction activity may be modulated by factors, such as calmodulin, that regulate channel permeability by binding to channel subunits such as MIP26 (Welsh *et al.*, 1982; Girsch and Peracchia, 1985; Peracchia and Girsch, 1989).

Finally, intercellular communication, between lens fibre and lens epithelial cells during development, has been investigated (Bassnett *et al.*, 1994), but this was not shown to be mediated by gap junctions, suggesting that there might be only limited communication between lens epithelial and lens fibre cells.

6.6. Cell adhesion molecules and integrins

A number of CAMs are expressed during lens development, including N-CAM, and cadherins, including A-CAM. N-CAM is expressed in the developing chick lens (Watanabe *et al.*, 1989). Its expression diminishes during lens epithelial cell differentiation, in contrast to the gap junction-associated protein MP26, which becomes more abundant. *In* culture, the addition of anti-N-CAM antibodies results in a reduced rate of lens epithelial cell differentiation, as judged by the extent of elongation of lens epithelial cells in transplanted epithelia. Furthermore, lens epithelial cell N-CAM was found to be more highly polysialylated in adult lenses than embryonic lenses, thus N-CAM could influence the formation of gap-junctions and adherens junctions through its degree of polysialylation (Watanabe *et al.*, 1992). A-CAM is a Ca^{2+}-

dependent protein expressed in a number of tissues, including differentiating chick lens epithelial cells and is involved in intercellular adhesion through adherens junctions (Volk and Geiger, 1986a;b). Finally, N-cadherin has been shown to be expressed in the developing chick embryo lens (Hatta *et al.*, 1987), although further studies do not appear to have been carried out to elucidate its specific function in the lens.

Integrins are a family of cell surface receptors that mediate cell-ECM interactions during physiological homeostasis and development (Hynes, 1992) and their importance for the early events of chick lens development has been illustrated by the study of Svennevik and Linser (1993) in which antibodies to integrins and an inhibitory peptide (RGD) were microinjected into the preoptic regions of chick embryos. The observed inhibition of the formation of ocular structures included complete inhibition of optic vesicle formation and its failure to completely separate from the head ectoderm, implying the importance of integrins for early eye morphogenesis. At slightly later stages of development, β_1 integrins have been shown to have a unique distribution in the chick lens (Menko and Philip, 1995) and are expressed in the lens fibre cells at their lateral borders and in their sites of attachment to the lens capsule. Furthermore, α_3 and α_6 integrins had a differential expression pattern in the lens epithelium and the lens fibres. Thus, it was concluded that the interaction of lens cells with their basement membranes, via integrin receptors, might be important in both signalling lens epithelial cell differentiation and maintaining the structural integrity of the lens.

6.7 Extracellular matrix

The ECM contributes to successful eye development at early stages by increasing the adhesivity between the lens primordia and the optic vesicle during induction, before invagination commences (Wakely, 1977; Hilfer, 1983). It has been demonstrated that this appears to be due to cross linking and polymerisation of optic vesicle associated glycosaminoglycans (GAGs) to ectodermal associated glycoproteins at early stages and by GAG replacement by collagen at later stages (Webster *et al.*, 1983). Fibronectin and laminin have also been localised to the eye during rat lens morphogenesis (Parmigiani and McAvoy, 1984) and were found in the basement membranes of ocular epithelia at all stages of rat lens differentiation. It was suggested that, in addition to providing good adhesion between the lens

placode and optic vesicle, the laminin- and fibronectin-rich ECM may stimulate the formation of basal extensions and cytoplasmic processes, from the presumptive lens ectoderm, which may contribute towards formation of the lens placode. In the mouse, the expression of the ECM proteins entactin and several laminin isoforms has been described (Dong and Chung, 1991) and it was revealed that these ECM components can be synthesised by multiple cell types and that the temporal expression of individual ECM molecules is tailored to the specific developmental requirements of individual structures in the developing lens and eye. Recently, a number of ECM components including laminin, collagens I and IV, fibronectin and GAGs have been localised during development of the macaque eye, a system which may be valuable because it is morphologically identical to human eye development (Peterson *et al.*, 1995).

The importance of the ECM for early eye development is illustrated by the aetiology of the developing eye in myelencephalic blebs (*my*) mice (Center and Polizotto, 1992). In these embryos, the normal histochemistry involved in the development of the eye is disrupted and this appears to be due to irregular deposition of GAGs in the ECM, between the optic vesicle and the ectoderm, and to increases in the amount of laminin in basement membranes. Trisomy 1 mice have a triplicated chromosome and they have also provided a useful model to determine if changes in ECM structure contribute to eye defects (Smith, 1989). Indeed, differences in ECM staining patterns were noted in the eyes of these mice compared to controls and there was an increased period of contact between the lens placode and optic cup, due to a persistence of the ECM in this region, which resulted in mice with abnormally shaped lenses. Thus, again, this illustrates how the spatial organisation of the ECM is important in the overall development of the eye and, more specifically, the lens.

The relative proportions of laminin and fibronectin in the lens capsule (the basement membrane surrounding the lens) may also be important in the regulation of lens epithelial cell migration during lens development (Parmigiani and McAvoy, 1991). This proposal was tested using lens epithelial explant cultures in which lens epithelia, from different stages of rat lens development, were transplanted on to laminin or fibronectin substrata. It was revealed that lens cells from all ages of rats migrated on laminin, while there was an age-related loss in the ability of these cells to migrate on fibronectin. Interestingly, this reduction in the ability to migrate on fibronectin was reflected in the developmental loss of fibronectin, at later stages of rat lens

morphogenesis, *in vivo*. Thus, both laminin and fibronectin appear to be important for promoting rat lens epithelial cell migration at early stages, while, at later stages, laminin is the key molecule promoting cell migration on the lens capsule. Similar results were reported by Olivero *et al.* (1993) who carried out a similar study using rabbit lens epithelial cells. Furthermore, these authors speculated that fibronectin deposition in the adult eye following surgery could re-activate posterior lens epithelial cell migration and, thus, could contribute towards the formation of posterior capsular opacification or secondary cataract. Furthermore, since growth factors effect a number of developmental events during eye development (Tripathi *et al.*, 1991a; see above), it is feasible that factors that influence ECM deposition and remodelling may contribute towards cataract development. For example, it has been suggested that since TGFß regulates ECM accumulation in the lens, during development, it could also have a role in subcapsular cataract formation (Liu *et al.*, 1994).

Finally, the ECM may have a role as a reservoir for growth factors with important roles in lens morphogenesis (see above), such as FGF, which can be found bound to heparan sulphate proteoglycans in ocular basement membranes, including the lens capsule (de Iongh and McAvoy, 1992).

6.8. The cytoskeleton

Lens fibres have an elaborate cytoskeletal structure and the crystallin proteins, themselves, may form a cytoskeletal structure that is unique to the lens (Piatigorsky, 1981). The cytoskeleton has an important role in co-ordinating the structural changes and biochemical events that occur in various parts of the cell (Ingber *et al.*, 1994) and the importance of cytoskeletal remodelling and integrity for lens development and function has been illustrated by several studies.

Alterations in lens fibre Ca^{2+} levels can modulate proteinase activities, which can influence the degradation of cytoskeletal components in lens fibres, such as actin, vimentin, and spectrin (Roy *et al.*, 1983; Yoshida *et al.*, 1984; Truscott *et al.*, 1989) during lens fibre cell terminal differentiation or cataract development. Indeed, a connection between cataract development and the disappearance of spectrin and vimentin from the cortex of the lens is provided by Truscott *et al.* (1990) who have shown that proteolysis, by Ca^{2+}-dependent

enzymes such as calpain, contributes to alterations in lens cell metabolism, cytoskeletal regulation, and the opacification associated with cataract.

Transgenic techniques have also been used to investigate the roles of cytoskeletal components in the developing lens. Vimentin and desmin are intermediate filament proteins that are expressed in the lens. Overexpression of desmin results in reduction in lens fibre nuclei denucleation, changes in cell shape, fibre fusion and alters the dynamics of cell membrane and cell junction turnover (Dunia *et al.*, 1990). Similar effects were observed in transgenic mice overexpressing vimentin (Capetanaki *et al.*, 1989); the denucleation process was again impaired, cell elongation was inhibited and the animals developed pronounced cataracts. Thus, the correct expression, assembly and membrane interaction of intermediate filament proteins would appear to be important in lens fibre cell terminal differentiation and alterations in these processes may contribute towards the formation of cataracts.

Recently, the possibility that vimentin contributes to the maintenance of the nucleus, in lens fibre cells in which it is expressed, has been investigated by Sandilands *et al.* (1995a). Vimentin is usually lost from lens fibres as they differentiate and lose their nuclei, while another type of intermediate filament structure in the lens, the beaded filament, composed of two proteins, CP49 and filensin, is present at all stages of lens fibre differentiation. However, Sandilands *et al.* showed that loss of vimentin was, in fact, a dramatic consequence of nuclear loss, and that reorganisation of the beaded filament network contributed to alterations in nuclear chromatin structure. This observation is supported by transgenic studies in which vimentin was knocked out and there was no effect on lens differentiation (Colucci-Guyon *et al.*, 1994). Rather, it would appear, from the transgenic studies described above in which desmin and vimentin were overexpressed, that there is significant functional redundancy among intermediate filament components and the relative proportions of intermediate filament components is more important in lens development than the affect of an individual cytoskeletal component. Furthermore, processing of filensin can result in different filensin fragments, which have distinct subcellular localisations in the peripheral and central lens fibres (Sandilands *et al.*, 1995b) and may, therefore, have different functional activities in the regions in which they are expressed.

Intermediate filament associated proteins (IFAPs) may also have important roles in lens development. Lieska *et al.* (1991) have described the expression and distribution of an IFAP that is associated with vimentin in the bovine lens. It has a differential distribution in the lens, being expressed in the fibre cells of the lens cortex, where it is associated with complexes near the plasma membrane, but not the central lens fibres.

Finally, in the newt, the lens can regenerate by a process of transdifferentiation of pigmented epithelia cells into lens fibre cells and this provides an ideal model system in which to study cytoskeletal changes occurring during lens fibre differentiation. Yang and Zalik (1994) have recently shown that this process is accompanied by reorganisation of cytoskeletal components, including smooth muscle-specific γ actin, muscle actin, and cytokeratin II. Interestingly, actin is expressed at the peripheral boundaries of elongating cells in their basal and apical regions, suggesting that actin could have a role in lens fibre cell elongation in addition to microtubules (Piatigorsky, 1972a; b).

6.9. Programmed cell death and lens fibre denucleation

Programmed cell death (see Chapter 3) occurs during the earliest stages of lens development and in the lens epithelial cells, while the terminal differentiation of the lens fibres involves the co-ordinated loss of cell organelles and the degradation of nuclei and is accompanied by DNA fragmentation.

6.9.1. Programmed cell death during early stages of lens development

Programmed cell death is important during early stages of lens development and occurs during detachment of the lens rudiment from the ectoderm in the chick embryo (Garcia-Porrero, 1979) and in the optic cup (Garcia-Porrero *et al.*, 1984), although, this may vary from the exact patterns of cell death observed during lens development in amphibians and mammals. It was suggested by Garcia-Porrero *et al.* (1979) that phagocytosis of dead cells is carried out by neighbouring healthy epithelial cells, but more recent evidence suggests that macrophages, from the haematopoietic lineage, invade the lens vesicle-ectodermal interspace, during closure and detachment of the avian lens, and it is these that are involved in the phagocytosis of dead cells (Cuadros *et al.*, 1991; Martin-Partido *et al.*, 1991).

6.9.2. Programmed cell death in the lens epithelium

It has only recently been revealed that the programmed death of lens epithelial cells is a significant component of lens development (Ishizaki *et al.*, 1993). Dying cells were found within the anterior epithelium of the rat lens and it was shown that lens epithelial cells, in serum-free culture, do not require other cell types for their survival and undergo apoptosis when cultured at low density. Cells could be rescued from death in low density cultures by the addition of conditioned medium from high density cultures, suggesting that lens epithelial cells produce a soluble survival factor(s). In bovine epithelial cells, the addition of aFGF antisense primers, which inhibit endogenous aFGF expression, leads to the death of these cells (Renaud *et al,* 1994), implicating aFGF as a survival factor in the developing lens. Furthermore, Robinson *et al.* (1995b) have demonstrated, using transgenic mice expressing truncated FGFR1, that FGF suppresses apoptosis in lens epithelial cells through FGFR1, since there was a diminished number of lens epithelial cells in these transgenic mice.

A greater understanding of the factors that regulate lens epithelial cell apoptosis is useful, since enhanced apoptosis in the lens epithelium is a common cause of non-congenital cataract (W. Li *et al.*, 1995a;b).

6.9.3. Denucleation of primary and secondary lens fibres

In the lens fibre cells, death, as defined by a loss of cellular structure and function, does not occur. However, the lens has provided an excellent model for chromatin degradation studies (reviewed by Counis *et al.*, 1989a) and the characteristic changes that occur, in lens fibre nuclei differentiating both *in vitro* and *in vivo* (see Fig. 6-4), resemble, in many respects, those occurring during apoptosis (see Chapter 3).

Primary lens fibres are formed by the elongation of lens epithelial cells at the posterior of the lens vesicle (Fig. 6-2). In primary lens fibres, denucleation occurs and is accompanied by the accumulation of small granules, which are probably nucleosomes and which condense to osmiophilic bodies in the nucleus and the cytoplasm. The osmiophilic bodies lie next to the cell membrane and become invaded by vesicles containing proteolytic

enzymes. The breakdown products are extruded into the extracellular space and transported to the anterior and posterior poles of the cells where they are digested and finally extruded from the lens (Vrensen *et al.*, 1991). This process is different from that occurring in secondary lens fibres.

Secondary lens fibre cells are formed by the addition of equatorial epithelial cells to the posterior compartment of the lens through cell division (Fig. 6-2). In secondary lens fibres, nuclei degenerate with a strict spatio-temporal pattern (Modak and Perdue, 1970; Kuwabara and Imaizumi, 1974) and a model for the transcriptional control of terminal differentiation in secondary lens fibre cells, proposed by Modak (1972), is still of use. In the chick embryo, the loss of DNA begins in the central lens fibres at ED 6. Appleby and Modak (1977) showed that DNA fragmentation, between nucleosomes, occurs in differentiating lens fibres and, when DNA from these fibres is run on a gel, the pattern obtained is reminiscent of the classic DNA ladders that accompany apoptotic cell death. The degeneration of nuclei is revealed by a characteristic pattern of morphological changes (pycnosis) that, in many respects, resembles the apoptotic morphology (Modak and Perdue, 1970). Pycnotic nuclei are apparent in the central lens fibres by ED 8. As development proceeds, there is an increase in the number of pycnotic nuclei and the wave of pycnosis spreads peripherally (Fig. 6-3; 6-4).

The number of DNA single-strand breaks increases in lens fibre cell nuclei as they degenerate (Modak and Bollum, 1972) and this could be due to a reduced efficiency of DNA repair in these cells (Counis *et al.*, 1977; 1979). Modak and Bollum (1970) showed that, during nuclear pycnosis of lens fibre cells from newly hatched chicks, lens fibre DNA undergoes single-strand breaks with the release of 3'-OH ends, which can then act as templates for terminal transferase in the nick-end tailing reaction (equivalent to TUNEL; see Chapter 5). However, lens fibre cell nuclei had no similar template activity for DNA polymerase in the nick translation reaction (Modak *et al.* 1969). This latter result has recently been verified in chick embryos by Chaudun *et al.* (1994) who suggested that, despite the presence of DNA strand breaks indicated by $(ADP\text{-}ribose)_n$ accumulation, the lack of nick-translation activity may be due to the accumulation of DNA breaks with 3'-OH ends blocked by phosphate groups. When

the phosphatase activity of nuclease P1 was used on lens fibre cells, incorporation of radiolabelled nucleotides into DNA was enhanced using DNA polymerase. However, these authors did not carry out similar experiments using terminal transferase. However, DNA strand-breaks in central lens nuclei from chick embryo lenses at ED 15 can incorporate labelled nucleotides using the TUNEL method (see Chapter 5), which utilises terminal transferase (Fig. 6-4c).

Chromatin condensation is a defining feature of pycnosis in secondary lens fibre cells (Sanwal *et al.*, 1986) and this may be due to alterations in histone deposition and metabolism (Roche *et al.*, 1992), which may, for example, be due to phosphorylation of specific nuclear substrates such as histones (Gao *et al.*, 1995). Chromatin condensation may affect the binding of endonucleases, which have been shown to have an important role in lens fibre DNA degradation (Counis *et al.*, 1989b). Two molecules, of 30 and 40 kDa, have been identified, which could be involved in the creation of nucleosome-size fragments during lens fibre terminal differentiation (Counis *et al.*, 1991). More recently, DNAse II has been shown to have a specific involvement in this process (Torriglia *et al.*, 1995). However, there is still no idea as to the identity of factors that may be involved in the induction of DNAse activity in these cells. Because TNFα induces DNA fragmentation by stimulating endonuclease activity in sensitive cells (see Chapter 3) and TNFα immunoreactivity is present in the developing chick embryo lens, just prior to the time when nuclear degeneration is occurring (see Chapter 4), TNFα could be involved in this process. This possibility is investigated in Chapter 7.

Transgenic techniques have also been used to investigate factors that may influence nuclear degeneration in secondary lens fibres. Oncoproteins, such as the E6 and E7 proteins from the type 16 papillomavirus, affect cell growth control through inactivation of cellular gene products, such as the retinoblastoma tumour suppresser gene and p53, a protein that mediates the induction of apoptosis by oncoproteins from DNA tumour viruses. Transgenic mice have been generated in which the expression of E6 and E7 is targeted to the lens by the αA-crystallin promoter (Pan and Griep, 1994). Both proteins produced lens defects, but E6 specifically inhibited lens fibre denucleation and the DNA fragmentation associated with this. It

was subsequently demonstrated that inhibition of lens fibre denucleation, by the E6 protein, occurred through p53-independent pathways (Pan and Griep, 1995). It is of interest, in the light of these transgenic studies describing the expression of viral proteins in the developing lens, that it has been known for a number of years that rubella virus infection, of mothers at early stages of pregnancy, results in a number of eye defects in the foetus, including congenital cataract (Vermeif-Keers, 1975; Armstrong, 1992). It is possible that this manifestation of rubella virus infection could be explained, at least in part, by the effects of viral proteins on the inhibition of naturally occurring p53-independent mechanisms of lens fibre cell denucleation occurring during development.

6.9.4. Organelle breakdown in secondary lens fibres

Nuclear degeneration is accompanied by the breakdown of cell organelles in lens fibres, including the mitochondria, Golgi apparatus, and endoplasmic reticulum (Bassnett, 1992; 1995). For example, Bassnett and Beebe (1992) have shown that, by ED 12 in the chick, the cells at the centre of the lens lack both mitochondria and nuclei. This disappearance is not due to a non-specific degradation of all intracellular structures, since the actin cytoskeleton remains intact, but rather may involve ubiquitin, an enzyme involved in proteolytic degradation (Hochstrasser, 1992; Ciechanover, 1994). Indeed, ubiquitin and ubiquitin conjugates have been identified in human lenses (Jahngen-Hodge *et al.*, 1992) and chick embryo lenses (Scotting *et al.*, 1991) and it was speculated that ubiquitin may have a role in lens differentiation. Furthermore, the recent identification of glutamate decarboxylase and γ-amino butyric acid in rat embryo lens fibres, and its possible relation to polyamine synthesis and nuclear and organelle breakdown, deserves further attention (X. Li *et al.*, 1995).

It is important to gain a greater understanding of the mechanisms of nuclear and organelle breakdown in the lens, since disturbances in this process may contribute to several pathological conditions including hereditary and congenital cataracts.

6.10. Pax and homeobox genes

Recently, attention has been drawn to the expression and potential roles of homeobox genes (Krumlauf, 1994; Beebe, 1994) and Paired box (*Pax*) genes (Gruss and Walther, 1992) in lens development. *Pax-6* is a gene that contains both paired box and homeobox motifs and it is expressed specifically in the developing brain and eye. Glaser *et al.* (1992) tested the hypothesis that, since the mouse mutation *Small eye (Sey)* results from defects in *Pax-6*, the corresponding human disorder, aniridia (disturbed development of lens, iris, cornea and retina), could result from defects in the human *Pax-6* gene. Indeed, it was shown, by these authors, that the human aniridia and mouse *Sey* phenotypes result from mutations to *Pax-6*. A similar mutation to *Pax-6* was also identified in the rat *Small eye (Sey)* mutant (Matsuo, 1993), in which lens induction was completely absent. *Pax-6* appears to be important in several processes during lens formation, which can be inferred from studies on *Sey/Sey* mice (Grindley *et al.*, 1995), including determination of the lens placodes from the surface ectoderm, and in maintenance of its own transcription in surface ectoderm domains that are independent of the optic vesicle. Further evidence of a role for *Pax-6*, in the determination of the lens, has been obtained by Li *et al.* (1994) who demonstrated that *Pax-6* is expressed at early stages of chick embryo development in regions of the future head ectoderm that are destined to give rise to the lens placodes. During invagination, the lens placodes also express high levels of *Pax-6* mRNA.

Cvekl *et al.* (1994) have shown that *Pax-6* is one of the transcriptional factors involved in the lens-specific induction of αA-crystallin, in the chick lens, and its repression in fibroblasts and that high αA-crystallin expression, in the mouse lens, is a result of synergy between *Pax-6* and cAMP response element binding (CREB) proteins (Cvekl *et al.*, 1995a). Furthermore, *Pax-6* is also involved in the lens-specific transcription of the δ-crystallin gene in the chick (Cvekl *et al.*, 1995b) and is also essential for lens-specific expression of guinea pig ξ-crystallin (Richardson *et al.*, 1995). Finally, *Pax-6* is expressed during urodele eye development and lens regeneration (Del Rio-Tsonis *et al.*, 1995).

So far, little attention has been paid to the presence of homeobox genes in the developing lens and the information that is available deals only with expression patterns, rather than with functions. Beebe (1994) has catalogued a number of homeobox genes that are

expressed in vertebrate ocular tissues. Only two genes, *msx-2 (Hox-8)* and *Emx-1 (empty spiracles)*, were cited as being expressed in the lens. Stadler and Solursh (1994) carried out *in situ* hybridisation to reveal the spatio-temporal expression pattern of GH6, a novel homeobox gene, in the chick embryo and its expression occurs in several craniofacial regions, including the lens epithelium. In addition, *Prox-1* (a mouse homeobox gene, which is homologous to the *Drosophila* homeobox gene *prospero*) is expressed in the developing mouse lens (Oliver *et al.*, 1993). Finally, SOX genes contain a homeobox motif and resemble the sex-determining factor *Sry* and are involved in lens-specific activation of δ1- and γF-crystallin genes in the mouse (Kamachi *et al.*, 1995). It will be of interest in the future to see if, as is the case with *Pax-6*, certain congenital lens malformations are associated with aberrant expression of homeobox genes.

6.11. Crystallins and crystallin gene regulation

6.11.1. Crystallins

Crystallins are the main structural components of the lens and contribute greatly to its optical properties (Piatigorsky, 1993). Furthermore, it has become apparent that crystallins are multifunctional proteins with close relationships to enzymes and stress proteins (de Jong *et al.*, 1989; Piatigorsky, 1993; Wistow, 1993). Indeed, α-crystallin is composed of two subunits, A and B, each of which possesses structural and functional similarities to the small heat shock protein (sHSP) family (Merck *et al.*, 1993). It has been proposed that the α-crystallin/sHSP family may have evolved to cope with conditions of cellular stress (de Jong *et al.*, 1993), and, furthermore, αB-crystallin is induced in lens epithelial cells in response to hypertonic stress (Dasgupta *et al.*, 1992) and is expressed during degenerative diseases (Boelens and de Jong, 1995). Expression of α-crystallin in the lens is advantageous to the organism, since α-crystallin is very stable, and like the sHSPs, can behave as a molecular chaperone in preventing undesirable protein-protein interactions and can assist in the refolding of denatured proteins (de Jong *et al.*, 1993; Caspers *et al.*, 1995). Furthermore, HSPs may also have a role in lens development, since HSP70 expression occurs in the developing chick lens and is associated with the differentiation of lens fibres (Dash *et al.*, 1994).

6.11.2. Crystallin gene regulation

The lens provides an ideal model for the study of factors that influence the sequential activation of tissue-specific genes during development, since the development of the lens is characterised by the expression of members of the crystallin family. This topic has been well reviewed on a number of previous occasions by Piatigorsky (1981; 1984; 1989; 1992), and by Kodama and Eguchi (1994), so that only some recent reports will be focused on here.

Inoue *et al.* (1992) quantified crystallin expression in the chick lens and showed that δ-crystallin accumulated rapidly during early development, composing up to more than 80% of total crystallins, while ß-crystallins accumulated significantly after hatching, and α-crystallin synthesis stayed at approximately 18% of the total throughout development. These data suggest that different regulatory mechanisms may work to influence the accumulation of each type of crystallin and this is dependent upon the stage of development of the lens.

It is apparent, from transfection and transgenic experiments, that transcriptional control by DNA regulatory elements in the 5' region is the primary basis for this tissue- and differentiation-stage-specific expression of crystallin genes (Piatigorsky, 1989). For example, two motifs of a 5' enhancer element of the chicken αA crystallin gene co-operate to confer lens specific expression of chicken αA-crystallin (Matsuo *et al.*, 1992). Similarly, in the mouse, αA crystallin synthesis requires the binding of the PE1 binding protein to the PE1 region, which is 5' to the αA crystallin gene (Sax *et al.*, 1995). In fact, a number of positive and negative regulatory elements bind proteins that regulate mouse and chicken αA-crystallin genes (Kantorow *et al.*, 1993a;b; Cvekl *et al.*, 1994), including Pax-6 (see above) and AP-1 (see Chapter 2), which contribute towards the high expression of αA-crystallin in lens cells and its low expression in fibroblasts. The importance of these 5' sequences for crystallin induction is highlighted by the study of Tomarev *et al.* (1994) revealing that the AP-1/antioxidant response element is a cis acting element involved in the induction of expression, during lens differentiation, of totally unrelated crystallin genes in the squid and the chicken; a case of convergent evolution.

Retinoic acid (RA) is known as an endogenous morphogen in development (Gudas, 1994) and has been shown to be involved in lens development, since overexpression of RA

binding protein I in the lens, under the influence of the αA-crystallin promoter, interferes with the development of the secondary lens fibres, causing cataract development (Perez-Castro *et al.*, 1993). Mouse γF-crystallin expression is regulated by several regulatory elements, including a proximal promoter region and two upstream enhancers (Goring *et al.*, 1993) and, furthermore, RA has a role in specific induction of γF-crystallin in the lens (Tini *et al.*, 1993). It has been demonstrated, by these authors, that the γF-crystallin promoter contains a RA response element, which is activated by the binding of RA receptors. Moreover, a more recent report, by the same group (Tini *et al.*, 1995), indicates that γF-crystallin expression, mediated by RA, can be further modulated by the binding of an alternative RA receptor, the RA receptor-related orphan nuclear receptor, to a hormone response element in the upstream enhancer region of the γF-crystallin promoter.

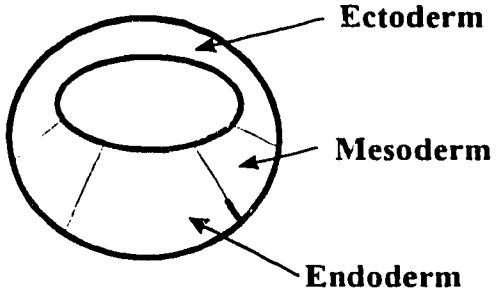
6.12. Concluding comments

It is apparent, from the literature reviewed here, that the developing lens is an excellent model system for the study of many cellular and molecular processes that are essential to successful embryonic development in general. It is also apparent that, as a result of this, lens development is well understood at both the cellular and molecular levels.

Since, from the work described in earlier Chapters of this thesis, it is apparent that TNFα is expressed in the developing avian lens and TNFα immunoreactivity appears to be associated with several tissues undergoing programmed cell death during development, Chapter 7 will focus on the developing lens as a model system to investigate more thoroughly the expression of TNFα, to investigate patterns of TNFR expression, and to analyse the potential effects of TNFα on lens fibre nuclear degeneration.

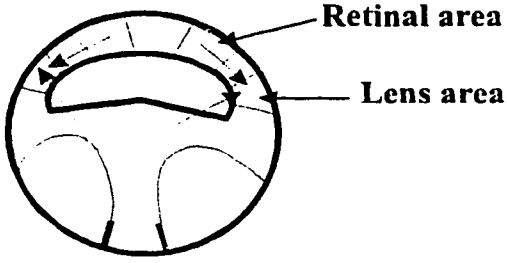
Figure 6-1. Current Model for Lens Induction. After Grainger (1992).
(Based on *Xenopus laevis*)

1. Early Gastrula (Pre lens competence)



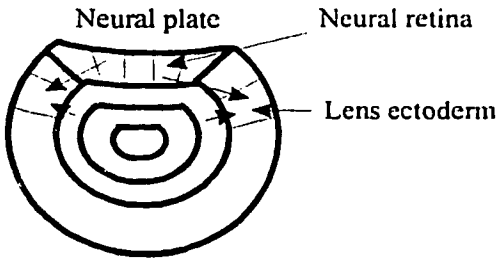
Ectoderm has yet to gain lens forming competence, but it does have neural competence.

2. Mid/Late Gastrula (Lens competence)



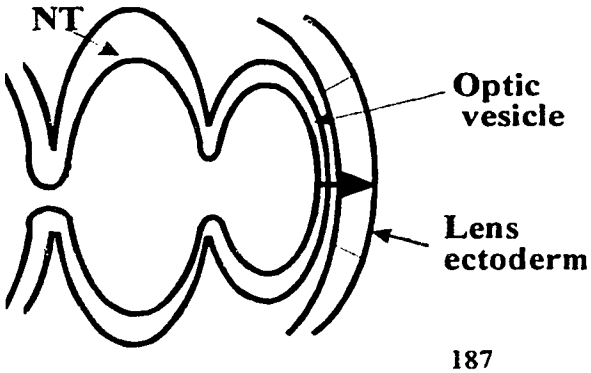
Mesoderm induces neural tissue, including the presumptive retina. Ectoderm has lens competence, but only briefly. Lens induction must commence during this time A planar signal from the retinal region, in the neural plate, provides the initial lens-determining stimulus. A region of endoderm, which will give rise to the foregut, is also involved.

3. Neural Plate Stage (Lens-forming bias)



Lens-inducing signals, from the neural retina, confer a lens-forming bias on the head ectoderm. This effect is enhanced by a signal from the underlying mesoderm.

4. Neural Tube (NT) Stage (Lens specification)

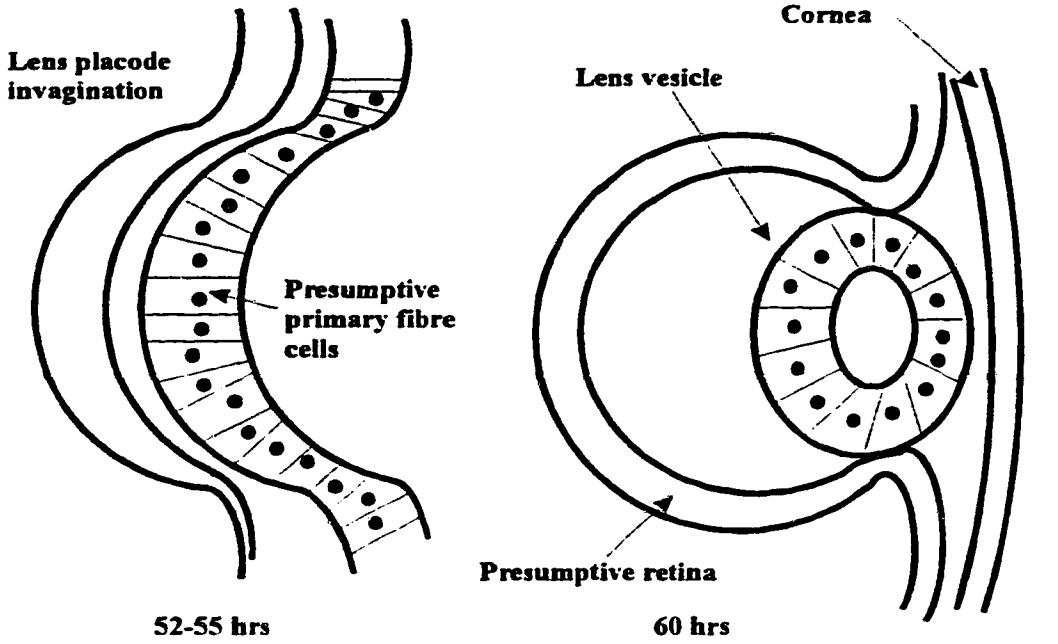


The optic vesicle comes into contact with the presumptive lens area and provides the final signal for lens determination, which results in specification of the lens ectoderm. Neural crest migration also begins at this stage and this helps to suppress the lens-forming bias in non-lens ectoderm.

Following lens specification, presumptive primary lens cell elongation begins in the presumptive lens ectoderm, thus forming the lens placode. This precedes lens placode invagination (See Fig. 6-2).

Figure 6-2. Stages of Lens Development Following Induction. After Piatigorsky (1981).

(Times refer to length of incubation in the chick embryo)



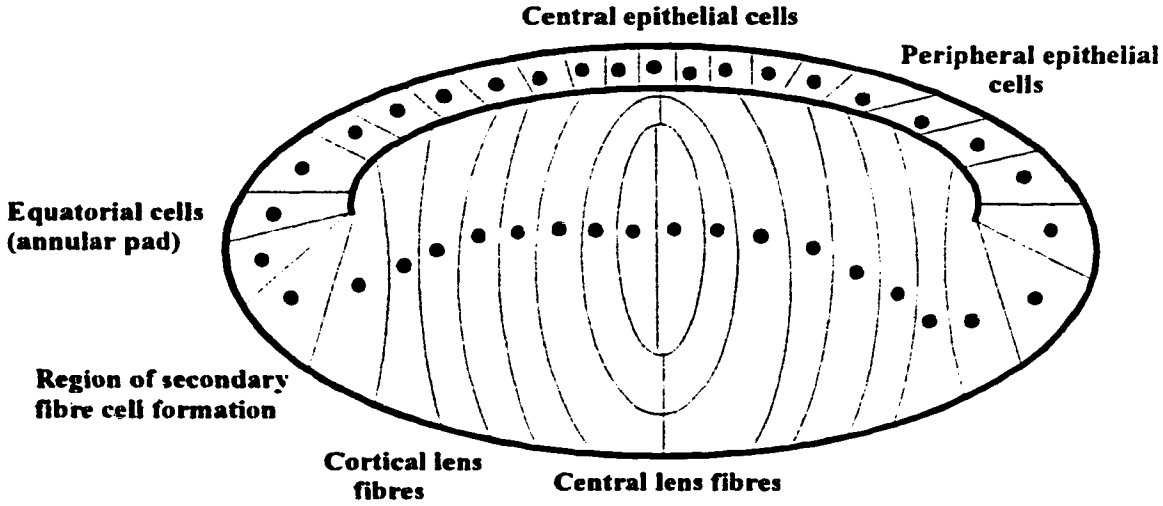
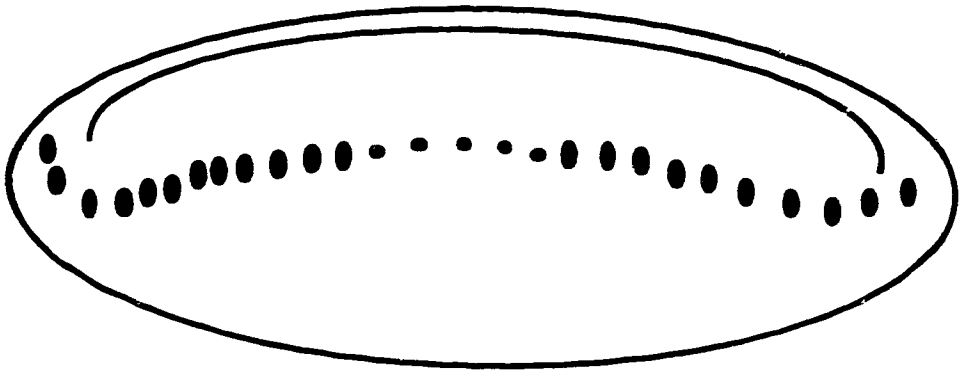


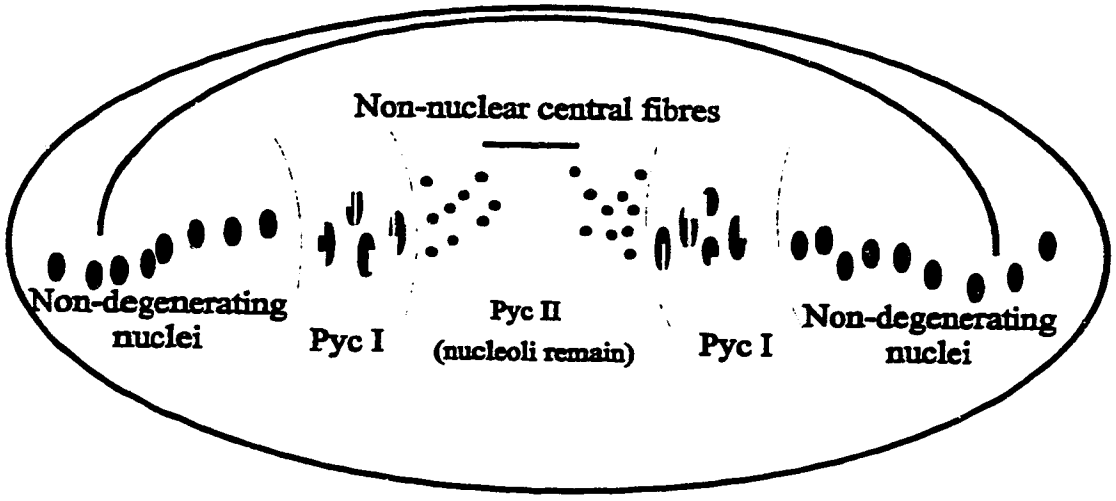
Figure 6-3. Patterns of Lens Fibre Cell Denucleation
During Chick Lens Development

8 day lens



Beginning of lens fibre denucleation at centre of lens
(Compare to 6 day lens, Fig. 6-2)

15 day lens



Lens fibre nuclei undergoing extensive degeneration:
Pyc I: nuclei in early stages of degeneration (pycnosis);
Pyc II: nuclei at late stages of degeneration

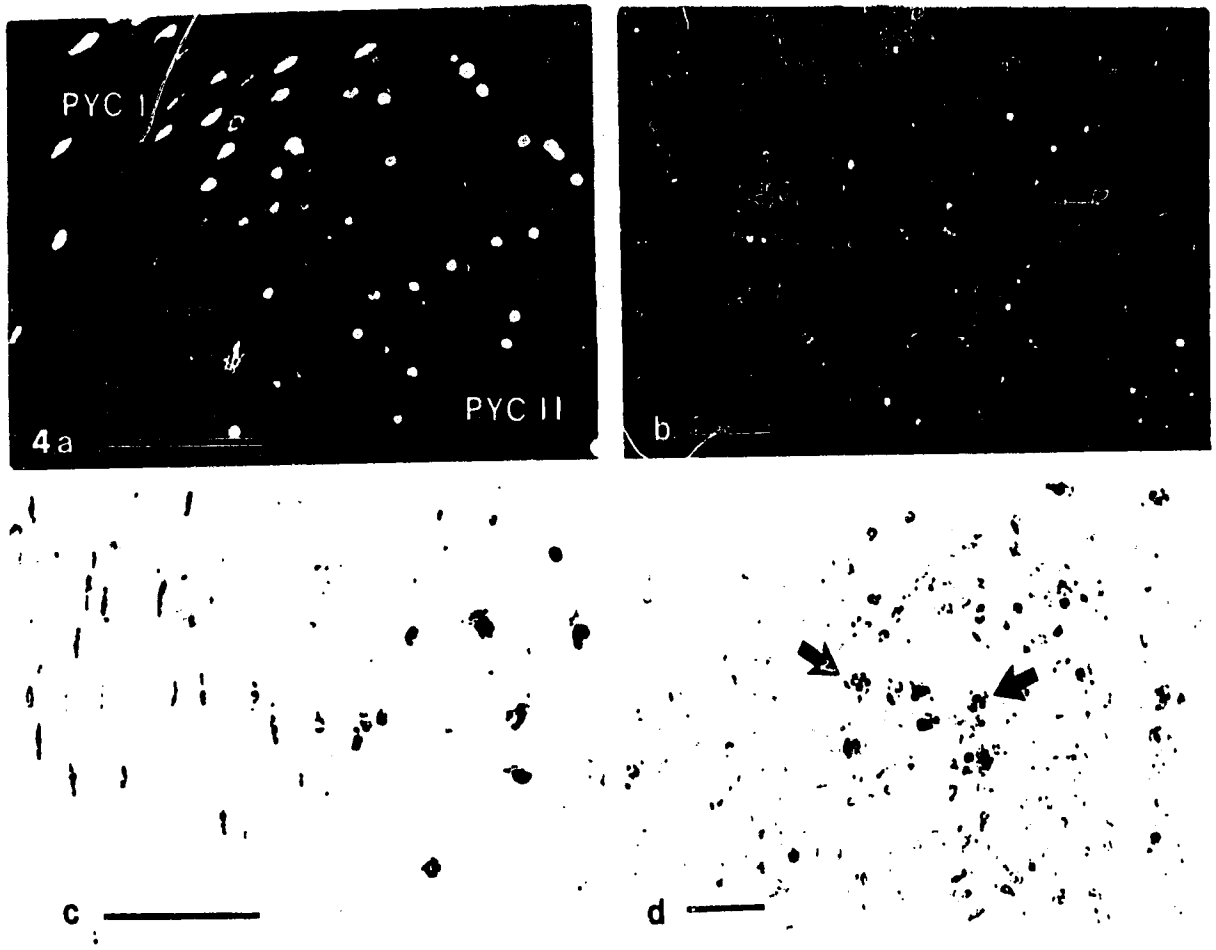


Figure 6-4. Pycnosis and DNA fragmentation in Lens sections and in lens cultures. (a). Section of embryonic day (ED 15) lens showing stages of nuclear degeneration (pycnosis) visualised using the DNA staining dye DAPI (Sigma), used at 2.5 μg/ml and applied to tissue for 10 minutes in the dark. PYC I: region of lens (cortical lens fibres) in which nuclei are in early stages of pycnosis (nuclei exhibit a morphology very similar to that present during apoptosis; see Chapter 4); PYC II: region of lens (central lens fibres) in which nuclei are in advanced stages of pycnosis (nuclei small and round). (b). PYC I nuclei, stained with DAPI (arrows), in chick lens epithelial cell culture (5 days of culture). The nuclei are in a higher focal plane than intact nuclei present in the epithelial monolayer (arrowhead). (c). Section of ED 15 lens stained using TUNEL (see Chapter 4). Nuclei are in various stages of pycnosis and also exhibit TUNEL labelling. (d). TUNEL labelling of degenerating nuclei in chick lens epithelial cell culture (5 days of culture). Bars = 50 μm.

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CHAPTER 7

TNFα AND ITS RECEPTORS IN THE CHICK EMBRYO LENS: EFFECTS ON DEGENERATION OF LENS FIBRE CELL NUCLEI[1]

7.1. Introduction

The lens is composed of two cell types, the epithelial cells located at the anterior of the lens and the fibre cells located at the posterior of the lens. The central lens epithelial cells are quiescent, while cells in the equatorial region of the lens undergo proliferation and elongation to form secondary lens fibres (Piatigorsky, 1981). Subsequently, lens epithelial cells differentiate into lens fibre cells by elongation, massive synthesis of specific crystallins, which are the structural components of the lens, and degradation of nuclei and organelles The cellular and molecular features of lens development have been well reviewed by Piatigorsky (1981) and recent studies have been reviewed here in Chapter 6.

TNFα is a highly pleiotropic molecule which may have multiple roles during embryonic development; for example, in programmed cell death; the regulation of cell proliferation; remodelling of ECM; and the control of expression of CAMs and integrins (See Chapter 3). In Chapter 4, it has been demonstrated that the lens fibres of the developing chick embryo have a particularly striking and consistent pattern of TNFα immunoreactivity at ED 4 (Stage 24-27; Hamburger and Hamilton, 1951). Furthermore, it was demonstrated in Chapter 5 that the expression of TNFα-like factors in the chick embryo occurs in some tissues in which cells are undergoing programmed cell death revealed by the TUNEL technique.

It is now recognised that programmed cell death is an integral component of morphogenesis in a wide range of developmental situations in both invertebrates and vertebrates (Sanders and Wride, 1995 and see Chapter 3). The fibres of the developing lens undergo what might be considered to be a specialised type of programmed cell death in that, although the lens fibre cells themselves are not removed, their terminal differentiation is

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[1] *A version of this Chapter is to be published. Part of this Chapter is in press. Sanders E J, Wride M A. (1996). Roles for growth and differentiation factors in avian development. Poultry Sci.*

accompanied by the degeneration of nuclei (including DNA fragmentation) and organelles in a precise spatio-temporal sequence (Modak and Perdue, 1970; Appleby and Modak, 1977; Piatigorsky, 1981; Bassnett and Beebe, 1992) and this resembles, in many respects, the morphology associated with apoptosis (see Chapter 6). The presence of TNFα immunoreactivity in known sites of programmed cell death has led to speculation that TNFα-like proteins could have a role in this process during embryonic development (see Chapters 3 and 4). It follows, therefore, that the presence of TNFα immunoreactivity in the developing chick embryo lens might suggest an involvement for TNFα-like proteins in the degeneration of nuclei in differentiating lens fibre cells and this Chapter, therefore, focuses on the potential effects of TNFα on lens fibre cell denucleation.

Furthermore, TNFα functional activity requires TNFRs (see Chapter 2). Therefore, it is important to determine if TNFRs are expressed in the developing lens and, if so, it is important to gain an understanding of their spatio-temporal patterns of expression. In addition, it is necessary to gain some understanding of the nature of their functional activities and of the nature of factors that may modulate the effects of TNFα on lens differentiation

In this Chapter, a combination of immunofluorescence and confocal microscopy has been used in order to reveal the patterns of TNFα, TNFR1, and TNFR2 immunoreactivity in the developing chick embryo lens, through a range of embryonic stages, as well as the patterns of bcl-2 and bax immunoreactivity. Bcl-2 is an oncoprotein that may modulate TNFα-mediated cytotoxicity (Hennet *et al.*, 1993) and bax belongs to the same family as bcl-2 and modulates its actions (Reed, 1994; Hockenbery, 1995). Also, the distribution of immunoreactivity for ICE has been investigated. ICE is a protease that is homologous to the product of the *C. elegans* cell death-promoting gene *ced-3* (Sanders and Wride, 1995) and appears to be involved in the TNFα-mediated cytotoxicity pathway (Enari *et al.*, 1995; Tewari and Dixit, 1995). In addition, using chick embryo lens epithelial cells, differentiating into lentoids (which resemble lens fibre cells) in culture (see Chapter 6; Menko *et al.*, 1984), it has been possible to investigate the effects of TNFα, anti-TNFα, and agonistic antibodies to TNFR1 and TNFR2 on lens fibre cell (lentoid) denucleation using image analysis.

The results suggest that TNFα may have a role in the denucleation of chick embryo lens fibre nuclei during embryonic development and that this effect is mediated by both TNFR1 and TNFR2. Furthermore, TNFα-mediated effects on lens fibre-denucleation could be influenced by the relative levels of oncoproteins such as bcl-2 and bax and could involve activation of ICE in lens fibre cells undergoing denucleation.

7.2. Material and methods

7.2.1. Preparation of lenses for immunofluorescence and TUNEL

White Leghorn hens' eggs were incubated at 37°C for 5, 8, 11 and 15 days (Stages 29-41; Hamburger and Hamilton, 1951). The embryos were removed from their yolk, rinsed and handled in Tyrode's saline and immediately decapitated with a sharp scalpel blade. Lenses were removed from the eyes of the embryos using electrolytically sharpened tungsten needles. Attached vitreous humour and retinal tissues were removed using sharpened forceps and tungsten needles. Lenses were fixed in 4% paraformaldehyde for 24 hrs (5 day lenses), 48 hrs (8 and 11 day lenses), and 72 hrs (15 day lenses), washed in PBS (pH 7.4), dehydrated through a graded series of alcohol, cleared in xylene and transferred to paraffin wax. Wax was allowed to infiltrate through the lenses for 48 hrs. Following this, lenses were embedded in paraffin wax and sectioned through their middle one third at 5μm thickness.

7.2.2. Immunostaining of sections

The polyclonal antibodies used were as follows: rabbit anti-murine TNFα (neutralising; Genzyme), rabbit anti-murine TNFR1 (agonistic; a gift from Genentech Inc.), rabbit anti-murine TNFR2 (agonistic; a gift from Genentech Inc.). Sections of lenses were de-waxed in Hemo-De (Fisher Scientific) and rehydrated through a graded series of ethanol. Sections were blocked for non-specific antibody binding for 30 mins in 2% BSA at room temperature. Excess BSA was then poured off and primary polyclonal antibodies added at a dilution of 1:200. Incubations were carried out for 1 hr at 37°C in a humidified chamber. Sections were then washed three times in PBS. The secondary antibody used for each of the above polyclonal antibodies was goat anti-rabbit IgG-Texas Red (Calbiochem) at a dilution of 1:50 in

1 % BSA in PBS for 30 mins in the dark at 37°C. Sections were again washed three times in PBS, coverslipped, mounted in Vectashield mounting medium for fluorescence microscopy (Vector Labs), and the edges were sealed with nail varnish. Immunoreactivity for TNFα on lens sections was revealed using an immunoperoxidase method in which the secondary antibody was goat anti-rabbit HRP (Calbiochem). This was applied to sections at the same dilution and for the same length of time as the FITC and Texas Red labelled secondary antibodies described above. Sections were quenched for endogenous peroxidase and staining was detected using the AEC method as described in Chapter 5.

7.2.3. TUNEL on lens sections

Labelling of fragmented DNA in lens sections was carried out as described previously (see Chapter 5) with some modifications. Lenses were incubated in 10 μg/ml proteinase K (Sigma) for 15 mins at room temperature; 0.3mM biotin-16-dUTP (Sigma) was mixed with the same volume of 1mM unlabelled dUTP (Sigma) in the reaction mixture and fragmented ends of DNA that had incorporated biotin-16-dUTP were detected using streptavidin conjugated fluorescein (Sigma) at a dilution of 1:50 in 1% BSA in PBS for 30 mins in the dark at 37°C.

7.2.4. Lens cultures

Lenses were cultured according to the method of Menko *et al.* (1984) with some modifications. Lenses were removed from 7.5-8 day embryos (stage 33; Hamburger and Hamilton, 1951), using sterile instruments, in Tyrode's saline containing antibiotics (Sigma; gentamycin and polymyxin B sulphate; 50 μg/ml each). Approximately 70 lenses were washed three times in Tyrode's saline containing antibiotics and then transferred to 1 ml of pre-warmed, sterile 0.1% fresh trypsin in calcium magnesium free (CMF) Tyrode's saline in an Eppendorf tube. The tube was incubated at 37°C for 15 mins. After this time, the tube was agitated every 5 mins for a further 15 mins. The tube was then centrifuged at 200g for 8 mins in an Eppendorf centrifuge and the supernatant removed. The pellet was then re-suspended by pipetting in 400μl of medium 199 (Gibco) containing 10% FCS (Gibco) and antibiotics (Sigma; gentamycin and polymyxin B sulphate; 50μg/ml each) and then filtered using a 40 μm

Falcon cell strainer to remove clumped fibre cells and capsular material and to obtain a cell suspension. Cells were counted using a haemocytometer and then plated in 30µl drops (approximately 4 x 10^5 cells per drop) at the centre of each of twelve glass coverslips in 35 mm Falcon dishes. Collagen IV (10µl; Collaborative Biomedical Research; 0.6 mg/ml) was spread and allowed to air dry onto each coverslip at the beginning of the experiment, to give an approximately 1 cm^2 area and then washed with medium 199 containing FCS and antibiotics. A thin film of Matrigel (Collaborative Biomedical Research; 1.2 mg/ml) was also an effective substrate for cell attachment (Ireland *et al.*, 1993) and this was applied to coverslips while ice cold. Subsequently, Matrigel was allowed to gel at 37°C for 30 mins and then washed with medium containing antibiotics, as above, before use. Incubation of cells was carried out in a humid atmosphere at 37°C in 5% CO_2. Cells were allowed to attach and begin to spread for 24 hrs when cultured on collagen IV and for 8 hrs when cultured on Matrigel. Lens cells differentiated well on collagen IV and Matrigel, although Matrigel provided a better substrate for cell attachment and spreading. Thereafter, the medium 199 containing 10% FCS and antibiotics was removed and the cultures washed three times in warm sterile Tyrode's saline containing antibiotics. This was designated day 0 of culture. Following this, 1ml of medium 199, containing bovine insulin (10µg/ml; Sigma) and gentamycin (50µg/ml), was added to the culture dishes. Subsequently, recombinant mouse TNFα (Genzyme) was added to give final concentrations of 10, 50, and 100 ng/ml; polyclonal anti-TNFα was added to give a final dilution of 1:250; and anti-TNFR1 and anti-TNFR2 agonistic antibodies were diluted 1:150 and 1:120 respectively to give a final concentration of 50 µg/ml in each case. Control experiments for polyclonal antibodies were performed by addition of the same dilutions of rabbit serum to the cultures and, in the case of anti-TNFα, also by pre-absorption of antibody with 50 ng/ml mouse TNFα (Genzyme). Medium was replaced every 48 hrs in all experiments, since TNFα has been shown to have a half life of 32 hrs in embryo culture medium (Lachapelle *et al.*, 1993).

7.2.5. Immunofluorescence on lens cultures

After 2, 4, and 6 days, cultures were washed in warm Tyrode's saline and fixed for 15 mins in 4% paraformaldehyde in PBS containing 0.5% Tween 20 (Sigma) and washed and stored in PBS at 4°C until processed for immunofluorescence. Cultures were stained for TNFα, TNFR1 and TNFR2 as described above. Staining for lens crystallins was performed using goat anti-chicken δ-crystallin antibody (a gift from J. Piatigorsky). Cultures were blocked for non-specific binding as described above. Antibody to δ-crystallin was used at a dilution of 1:100 for 30 mins at 37°C in a humidified chamber. The secondary antibody was Texas Red conjugated rabbit anti-goat IgG (Calbiochem). Controls were performed using the same dilution of pre-immune goat serum.

7.2.6. Double labelling for TUNEL and Immunofluorescence

Cultures were fixed as described above and TUNEL was carried out as described above and previously (see Chapter 5) except that no proteinase K digestion was used on cultured cells. Cultures were then stored in PBS at 4°C until processed for immunofluorescence as described above. Following addition of primary antibodies, fluorescent secondary antibodies (Calbiochem) and FITC conjugated streptavidin (Sigma) were diluted 1:50 together in 0.1% BSA in PBS and applied to sections and cultures for 30 mins at 37°C in the dark. Sections and cultures were then mounted as described above.

7.2.7. Immunofluorescent staining for bcl-2,bax, and ICE

Lens sections were stained for the cell death modulating oncoproteins bcl-2 and bax using a polyclonal rabbit anti-mouse/human bcl-2 antibody (N-19; Santa Cruz Biotechnology, Inc.), a polyclonal rabbit anti-mouse/human bax antibody (N-20; Santa Cruz Biotechnology, Inc.), and for ICE, a protease known to be involved in the cell death pathway, using polyclonal rabbit anti-mouse/human ICE antibody (M-20; Santa Cruz Biotechnology, Inc.). Sections were incubated with 1% BSA at room temperature for 30 mins and then anti-bcl-2, anti-bax, or anti-ICE were applied to sections overnight at 1:100 in PBS at 4°C. Sections were then washed three times in PBS and secondary antibody (goat anti-rabbit FITC; Calbiochem) was then applied at 1:50 in PBS plus 0.1% BSA for 30 mins at 37°C in the dark. Controls

consisted of sections incubated with non-immune rabbit serum at 1:100 or antibodies preabsorbed with a two-fold excess of control peptide, which was specific to the antibody used (Santa Cruz Biotechnology Inc.). Sections and cultures were then mounted in Vectashield as described above and viewed.

7.2.8. Confocal laser scanning microscopy

For lentoids in culture, it was not easy to obtain data by conventional photography, since lentoids have a complex three dimensional shape, which means that there is a lot of background fluorescence in these cultures. Therefore, to avoid the problem of fluorescence from different focal planes while photographing lentoids, a Leica confocal laser scanning microscope equipped with an argon/krypton laser, was used. A short pass excitation filter was used with the beam splitter in the neutral position. The barrier filter used in the Texas Red channel was OG530 and that in the FITC channel was OG550. Images from the two channels were collected sequentially as 16-scan averages. Some images were then digitally combined and artificially coloured to give final double-label images. Texas Red stained regions (δ-crystallin, TNFα, TNFR1, and TNFR2) were coloured red and FITC-labelled regions (TUNEL) were labelled green. Overlap of these two colours produced yellow.

7.2.9. PAGE and Western Blotting: Lenses were removed from embryos as described above and all lenses were homogenised in protease inhibitor buffer as described by Jaskoll *et al.* (1994). Protein concentrations were determined using the Bio Rad protein assay method and 8 μg of lens protein was added to each well of a 10% gel. Polyacrylamide gel electrophoresis and Western blotting were carried out as described previously (see Chapter 4) and bands were detected using enzyme chemiluminescence (Amersham).

7.2.10. Quantitative Western blotting for δ-crystallin in cultures

In order to determine if there was any effect of TNFα or anti-TNFα on δ-crystallin deposition in lens cultures, quantitative Western blotting was performed on lens epithelial cell culture lysates. Cultures were prepared as described above and plated on a thin film of

Matrigel coated on the bottom of a 35mm plastic petri dish (Falcon) and incubated with various concentrations of TNFα, anti-TNFα, or anti-TNFα preabsorbed with TNFα. Culture medium was changed every day. After 3 days of incubation, cultures were washed in warm Tyrode's saline and a 10 µl drop of cell solubilisation buffer was added to each culture and incubated for 1.5 hours at 4°C. Cell solubilisation buffer had the following composition: 0.05M TRIS, 5M urea, 0.0025% SDS, 20mM HEPES, 10mM $MgCl_2$, 2mM EDTA, 0.2mM EGTA, 15µg/ml aprotinin, 1µg/ml leupeptin, 5µg/ml pepstatin, and 1.74 mg/ml PMSF. Following incubation, cultures were scraped from the dish using a bent tungsten needle and pipetted into eppendorf tubes using a drawn out Pasteur pipette. Protein assays were performed on the samples as described above and 4 µg of protein was added from each lysed culture to each well of a 10% polyacrylamide gel. PAGE was carried out as described in Chapter 4. Western blotting was performed by transferring protein from the gel to a nitrocellulose membrane as described in Chapter 4. Antibodies to chicken δ-crystallin were applied to the membrane at a dilution of 1:750 overnight at room temperature while shaking. Following this, the membrane was washed as described in Chapter 4 and the secondary antibody (rabbit-anti-goat HRP; Calbiochem) was applied to the membrane for 2 hours at a dilution of 1: 10,000. Bands were detected using the ECL method (Amersham).

7.2.11. Image analysis

Lens cell cultures, stained with antibodies to δ-crystallin (Texas Red) and on which TUNEL (FITC) was carried out, were subjected to image analysis using the Universal Imaging Corporation Image Analysis system. For the different conditions to which the cultures were subjected, the number of TUNEL-positive nuclei per unit area of lentoids (red, δ-crystallin-positive areas) was determined. This was carried out by randomly selecting areas of the culture for analysis while viewing the red δ-crystallin staining using the green filter. To acquire an image, the computer was set to 200 integrations. The area of the δ-crystallin-positive region (area of lentoids) was then determined by thresholding the image in the "measure objects" mode. The image obtained was then loaded to the "background" page. While keeping the same field in view, the blue filter was used to reveal TUNEL-positive (green) nuclei in the same

region. An image was obtained after 20 integrations and this image was loaded to the "foreground" page. Using the manual object count device, the number of TUNEL-positive nuclei were counted by clicking on each one and thus marking them on the screen. Turning back to the "background" page, the distribution of TUNEL-positive nuclei could be seen superimposed on the area of δ-crystallin identified. Any TUNEL-positive nuclei falling outside the bounds of the δ-crystallin-positive area identified; i.e. not truly degenerating nuclei, but apoptotic cells in the epithelial monolayer (which were very few), were excluded from the count. This process was completed for each culture starting at the bottom left, moving towards the right and then returning to the left slightly above the line of the previous count. This ensured that the same region of the culture was not counted more than once, which would bias the results. Three cultures, subjected to the same experimental condition, were used to obtain values (see Appendix II), which could then be averaged. Analysis of data was carried out using the unpaired, two tailed student's t-test using the Microsoft® Excel spreadsheet programme to determine the significance of the differences between experimental conditions.

7.3. Results

7.3.1. Western blotting of lenses

i. TNFRs: Western blotting was carried out to investigate the expression of TNFR1 and TNFR2 during chick lens development. TNFR1, at its recognised molecular weight of 55kDa was detected. (Fig. 7-1a) and the intensity of bands diminished from ED 6 to ED 8. In a similar blot, probed with antibodies to TNFR2, no immunoreactive bands were detected (not shown).

ii. TNFα: Despite the fact that protease inhibitor buffer was used to homogenise lenses and that, using this buffer, a 17kDa TNFα immunoreactive band has been identified in Western blots of whole embryo homogenates (see Fig. 4-4), a 17kDa TNFα immunoreactive band was not detected in the lens at any stage examined here. At all other stages, a 70kDa immunoreactive band was the only one detected (Fig. 7-1b). Thus, the 70kDa reactive protein would appear to be the most predominant during lens fibre differentiation.

7.3.2. Immunostaining on sectioned lenses

i. TNFα: TNFα immunoreactivity was first detected in the lens fibres at ED 4 (see Chapter 4). Here, TNFα immunoreactivity was also revealed in the fibre cells of the ED 5 lens (Fig 7-2a). However, from ED 8 onwards, TNFα immunoreactivity was not detected in the cytoplasm of the lens fibre cells, but it became localised to the lens epithelial cells (Fig. 7-2b; ED 11) and this pattern persisted at ED 15 (data not shown). TNFα immunoreactivity colocalised with the TUNEL labelled nuclei of central lens fibres of the ED 15 lens (Fig. 7-6c).

ii. TNFRs: At ED 5, TNFR1 immunofluorescence was present in the lens fibres, but not in the nuclei of these cells, nor was it expressed in the lens epithelium at these stages (Fig. 7-3a). TNFR2 was not significantly expressed in the lens at ED 5 (Fig. 7-3b). At ED 8, TNFR1 immunofluorescence was present in the lens epithelium and the annular pad as well as in a diffuse pattern in the cortical fibre cells (Fig. 7-3c). Lens fibre cell nuclei were also positive for TNFR1 immunofluorescence at this stage (Fig. 7-3c). On the other hand, although TNFR2 has a similar pattern of labelling at ED 8, it was not present in the nuclei of either the epithelium or the cortical fibre cells (Fig. 7-3d). However, faint immunofluorescence for TNFR2 could be seen in the nuclei of the more central fibre cells (Fig. 7-3d). At EDs 11-15, the distribution of TNFR immunofluorescence was similar to that at ED 8, with only some subtle differences (data not shown). TNFR1 immunoreactivity persisted in the epithelium, but was not present in lens epithelial cell nuclei, although TNFR1 remained in lens fibre nuclei at ED 11. At ED 11, TNFR2 was identical to that seen at ED 8, while at ED 15, TNFR1 immunofluorescence was present in the lens epithelium as well as in cortical fibre cells, but not in their nuclei, while it persisted in the nuclei of more central fibre cells. TNFR2 was localised exclusively to the lens epithelium at ED 15 .

7.3.3. Immunofluorescence for TNFα, TNFR1, and TNFR2 on lens cultures

The differentiation of lentoids from lens epithelial cells in culture could be seen using phase contrast microscopy (Fig. 7-4a) and TNFα expression could be seen at the surfaces of these lentoids using conventional fluorescence microscopy (Fig 7-4b). However, for the

reasons described above, confocal laser scanning microscopy was subsequently used to localise immunofluorescence in lentoids.

i. δ-crystallin: Although present to some degree in the epithelial monolayer, particularly at the apical edges of cells, δ-crystallin was dramatically up-regulated in lentoids and this coincided with the initiation of DNA fragmentation in these lentoids (Fig 7-6a).

ii. TNFα: Lens epithelial cells showed immunoreactivity for TNFα, particularly at their surfaces (Fig. 7-5a). TNFα immunoreactivity was present in lentoids and the presence of TNFα within nuclei often coincided with DNA fragmentation detected by TUNEL (Fig 7-6b).

iii. TNFR1: TNFR1 immunoreactivity was present at the surfaces of lentoids and in their nuclei (Fig. 7-5d) and its expression coincided with TUNEL labelling in lentoids (7-6d). TNFR1 was also present in the nuclei of mesenchymal cells at the edge of the culture (Fig. 7-5e), and it was present at the lateral edges of cells in the epithelial monolayer and in lens epithelial cell nuclei (Fig. 7-5c).

iv. TNFR2: TNFR2 was expressed faintly in lentoid cell nuclei in some cases, where it coincided with TUNEL labelling (Fig. 7-6e) and was expressed in a punctate pattern at the lateral edges of cells in the epithelial monolayer (Fig. 7-5f).

7.3.4. Image analysis: TUNEL-positive nuclei per unit area of lentoid culture[2]

Analysis of the cultures of lens epithelial cells, under different conditions and after different lengths of incubation time, allowed a value for the number of TUNEL-positive nuclei per unit area of lentoid (δ-crystallin-positive region) to be obtained in each case. All experiments were performed using cultures grown on collagen IV unless otherwise stated.

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[2] *The raw data obtained here is presented in Appendix II, along with comprehensive tables of statistical analysis.*

i. Two days of culture: It is apparent from Fig. 7-7a (top) that the addition of TNFα to the cultures, for 2 days, at concentrations of 10 ng/ml ($p<0.05$), 50 ng/ml ($p<0.01$), and 100 ng/ml ($p<0.01$) all significantly enhanced the number of TUNEL-positive lentoid cell nuclei per unit area of lentoid compared to control cultures containing 10 μg/ml of insulin only. However, it is also noteworthy that, after 2 days, addition of TNFα to the cultures at 10 ng/ml ($p<0.01$), 50 ng/ml ($p<0.01$), and 100 ng/ml ($p<0.01$) had significant effects on the reduction of the mean lentoid area per measurement (Fig. 7-7a; bottom left)[3]. Fig. 7-7a (bottom right) reveals that there is no significant increase in the number of TUNEL-positive nuclei per measurement at 100 ng/ml TNFα after 2 days in culture when compared to the control. Thus, at early stages of lentoid differentiation, the major effect of TNFα appears to be on lentoid area, rather than on an increase in the number of TUNEL-positive nuclei. In contrast, the addition to the cultures of a polyclonal neutralising antibody to mouse TNFα significantly reduced the number of nuclei per unit area of lentoids (Fig. 7-7a; top) compared to control cultures containing insulin alone ($p<0.01$), non-immune rabbit serum at the same concentration ($p<0.01$) or antibody preabsorbed with TNFα ($p<0.01$). It appears that this effect was a consequence of a specific effect on the numbers of positive nuclei per measurement (Fig. 7-7a; bottom right) compared to rabbit serum and preabsorption controls ($p<0.01$).

In a different experiment, addition to the cultures of agonistic antibodies to TNFR1 or TNFR2 enhanced the number of TUNEL-positive nuclei per unit area of lentoids (Fig. 7-7b; top) compared to controls. Interestingly, in this experiment, there were no significant differences in the mean lentoid area per measurement compared to controls, but the mean number of nuclei per measurement was enhanced significantly using both antibodies ($p<0.01$; Fig. 7-7b).

ii. Four days of culture: After 4 days of exposure to different concentrations of TNFα, 50 ng/ml and 100 ng/ml of TNFα significantly enhanced ($p<0.01$) the number of

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[3] *In addition, increasing concentrations of TNFα reduced the mean % area of lentoids per culture (see Appendix II-3).*

TUNEL-positive nuclei per unit area of lentoids (Fig. 7-8; top) compared to control cultures. The maximum number of TUNEL-positive nuclei per unit area of lentoid was obtained with 50 ng/ml TNFα. The effect of 50 ng/ml of TNFα ($p<0.01$) and 100ng/ml of TNFα ($p<0.05$) on the reduction in average area of lentoids per measurement was also significant (Fig. 7-8; bottom left). In addition, the effect of TNFα at 50 ng/ml ($p<0.01$) and TNFα at 100 ng/ml ($p<0.05$) on the increase in the number of TUNEL-positive nuclei was significant (Fig. 7-8; bottom right). The effect of anti-TNFα observed in these cultures was solely due to its effect on reducing the numbers of TUNEL-positive nuclei ($p<0.01$) and there was no significant effect on the lentoid area per measurement compared to cultures with insulin alone (Fig. 7-8; top).

iii. Six days of culture: After 6 days of exposure of lens epithelial cell cultures to various concentrations of TNFα, it was revealed that there were significant effects of 50 ng/ml ($p<0.01$) on the number of TUNEL-positive nuclei per unit area of lentoids (Fig. 7-9; top). TNFα at 100 ng/ml was also significantly different ($p<0.01$), but this was because the number of TUNEL-positive nuclei was significantly less than that obtained with insulin alone (Fig. 7-9; top). After 6 days in culture, there were no significant differences in the average area of lentoids per measurement (Fig. 7-9; bottom left), implying that all the effects seen at this stage are due to an increase in the numbers of TUNEL-positive nuclei per measurement (Fig. 7-9; bottom right).

iv. Summary: The changing response of lens epithelial cells, differentiating in culture, to TNFα is seen in Fig. 7-10. As the cultures progress from 2 days to 6 days, the effect of TNFα on the reduction in lentoid area per measurement diminishes, while the effect on the increase in TUNEL-positive nuclei per measurement becomes more predominant.

7.3.5. Quantitative Western blotting for δ-crystallin in cultures

Cultures of lens epithelial cells were grown on Matrigel for 3 days, and incubated with different concentrations of TNFα and with anti-TNFα. There were no differences observed in

the intensity of bands detected in a Western blot of lens epithelial cell cultures, under the different conditions, when stained for δ-crystallin (Fig. 7-11).

7.3.6. Staining for bcl-2, bax and ICE

i. Bcl-2 staining: At ED 8, bcl-2 protein was expressed in the lens epithelium and in the lens fibres (not shown). By ED 15, bcl-2 staining was restricted to the lens epithelium, the annular pad region and was expressed at lower levels in the outermost lens fibres (Fig. 7-12). Control sections incubated with antibody preabsorbed with control peptide exhibited no staining.

ii. Bax staining: Bax staining was detected at ED 8 in the central lens fibre cells and in the lens epithelial cells (7-13a) and was present at the surface of the central lens fibre cells, but not in their nuclei (Fig. 7-13b). Control sections incubated with antibody preabsorbed with control peptide exhibited no staining.

iii. ICE staining: ICE immunoreactivity was associated with the lens epithelium and with cortical lens fibre cells and was detected in the central lens fibre cells in a punctate pattern at ED 8 (Fig. 7-14a; b). Control sections incubated with antibody preabsorbed with control peptide exhibited no staining.

7.4. Discussion

It has been shown here that DNA strand-breaks, in central lens nuclei from chick embryo lenses at ED 15 and of lens epithelial cells differentiating into lentoids in culture, can incorporate labelled nucleotides using the TUNEL method (Gavrieli *et al.*, 1992), which utilises terminal transferase. Therefore, TUNEL proved to be a useful method for labelling fragmented DNA in nuclei degenerating in lens fibres and lentoids.

DNases have been shown to have an important role in lens fibre DNA degradation (Counis *et al.* 1989). However, there is still no idea as to the identity of factors that may be involved in the induction of expression of DNases in differentiating lens fibres. In this Chapter, some evidence has been provided that factors that posses TNFα activity, may be involved in

this process, since TNFα induces DNA fragmentation by stimulating endonuclease activity (Larrick and Wright, 1990).

7.4.1. Western blot analysis of TNFα, TNFR1, and TNFR2

A TNFα cross reactive protein of 70kDa was expressed throughout the period of chick lens development studied here. The true nature of this protein remains uncertain, but it is likely that it represents a TNFα-like membrane bound factor, since in previous studies (see Chapter 4), it has been revealed, using immunogold labelling, that TNFα-cross reactive proteins, including a 70kDa protein, are expressed on the surfaces of cells in the gastrulating embryo. At no stage of lens development was 17kDa TNFα detected, suggesting that this soluble form of the TNFα molecule may not have a significant involvement in lens development or that it is present, but at concentrations below the level of detection by the methods employed. The intensity of TNFα-immunoreactive bands appears to diminish from ED 6-15 suggesting that, since the same concentration of lens protein was loaded to each well for each stage examined, the proportion of TNFα to totai lens protein becomes progressively lower as lens development proceeds. This is most likely due to the massive deposition of crystallin proteins occurring during the stages examined here.

Although a TNFR1 immunoreactive band was detected at the recognised molecular weight (55 kDa) in Western blots of lenses, TNFR2 was not detected. This lack of detection of TNFR2 is probably due to its expression below the level of detection in Western blot experiments, since overall it is less abundant during lens development, particularly in lens fibres, as judged by the immunolocalisation studies described here. Higher yields of chick TNFR2 might be obtained for western blotting by dissecting out and homogenising a region of the lens that is rich in TNFα immunoreactivity; for example, the annular pad region

7.4.2. TNFα and TNFR expression in developing lenses

i. Expression in lens fibres and lentoids: It is apparent from the present results that TNFα immunoreactive proteins are present within degenerating lens fibre cell and lentoid cell nuclei, suggesting that proteins with TNFα-like activity could have effects on

nuclear degeneration following their translocation to the nuclei of lens fibre cells. Panagakos and Kumar (1994) pointed out that TNFα has a nuclear localisation sequence and reported that following binding to its receptors on human osteoblast-like cells, TNFα is internalised and rapidly transported to the nucleus. A number of growth factors and cytokines have recently been shown to be translocated to the nucleus (reviewed by Prochiantz and Theodore, 1995). The effects of growth factors and cytokines in the nucleus are largely unknown, but could include modulation of mRNA transport and nuclear protein phosphorylation, activation of nuclear factors, and stimulation of mitosis (Panagakos and Kumar, 1994). In the present study, it would appear that the 70kDa protein is the only TNFα-related protein detected in the lentoid cell nuclei, since 17kDa TNFα was not detected at any stage examined. However, this does not rule out the possibility that 17kDa TNFα may be present in these nuclei, but at concentrations below the level of detection (see above).

TNFRs, particularly TNFR1, are also present within the nuclei of lens fibres in sections and lentoids in culture. This suggests that TNFRs could accompany, or facilitate the transport of, TNFα-like factors to the nuclei of lens fibres. TNFR1 appears to be more abundant in lentoids than TNFR2 and this is probably a result of its internalisation following TNFα binding, whereas TNFR2 expression in lentoid nuclei is a more difficult observation to explain, since TNFR2 is generally shed upon binding of TNFα (see Chapter 2). Moreover, it is also possible that some of the TNFR immunoreactivity detected in lentoids is a result of translocation of TNFR from sites of synthesis to the cell surface. A more detailed analysis of TNFR sub-cellular distribution in lens fibre cells would be useful in this regard. In addition, TNFR1 immunoreactivity has also been found associated with the nuclei of cells from gastrulation stage chick embryos (Sanders *et al.*, 1996).

ii. TNFα and TNFR immunoreactivity in lens epithelial cells *in vitro* and in sections: The presence of TNFα and TNFR immunofluorescence in lens epithelium in sections and in the lens epithelial cell monolayer in culture could reflect a role for TNFα-related molecules and TNFRs in some aspect of lens epithelial cell differentiation and/or proliferation (discussed below).

7.4.3. Image analysis

i. TNFα: The effect of TNFα on lens epithelial cell differentiation into lentoids in culture was two-fold. During the first two days of culture, its effects on reducing lentoid area predominated over its effects on enhancement of nuclear degeneration, whereas by 6 days in culture the effects of TNFα on nuclear degeneration predominated. It has been demonstrated that chick lens epithelial cells, particularly those from the annular pad region, are highly proliferative when initially plated, but lose their proliferative potential as they differentiate (Menko *et al.*, 1984) and it was also shown that it is the highly proliferative cells that differentiate into lentoids. It follows that any factors that are capable of inhibiting lens epithelial cell proliferation will inhibit the number of cells available to differentiate first into lentoids and this will, therefore, result in a reduction in lentoid area in the cultures. TNFα has effects on cell proliferation (see Chapter 3) and is cytostatic to many cell types, particularly during haematopoiesis (see Chapter 2). Furthermore, it has been demonstrated here that TNFα is expressed in the annular pad cells and it is in this region that rates of lens epithelial cell proliferation are tightly regulated in order to control the number of cells moving into the posterior of the lens and, therefore, the number differentiating into lens fibre cells (Harding *et al.*, 1971). This process is controlled by a balance between factors that promote proliferation in the lens epithelium; for example, PDGF may have a role (Potts *et al.*, 1995), and factors that inhibit proliferation in the lens epithelium. Therefore, it is possible that TNFα may have a role in inhibiting the rate of lens epithelial cell proliferation, since it is expressed in the lens epithelium and annular pad and, therefore, the number of cells entering the lens fibre compartment of the lens and the number of cells entering lentoids in culture. This would also explain why, as differentiation proceeds in culture, the effect of TNFα on lentoid area becomes less significant compared to its effects on lentoid cell denucleation, since lens epithelial cells lose their proliferative potential as differentiation becomes the predominant process, with increasing time in culture. Studies are currently being performed to further investigate the possible effects of TNFα on lens epithelial cell proliferation in culture.

It is also possible that the observed effect of TNFα on the reduction of lentoid area could be a result of an effect of TNFα on lens fibre cell elongation, since if, as the lens fibre

cells elongate, they maintain their volume, it follows that their cross sectional area would be reduced. However, this is probably not the case, since lens fibre cells actually increase in volume during elongation (Piatigorsky, 1981) and thus probably maintain a near constant cross sectional area during this process.

After 6 days of culture, 50 ng/ml gave the maximum mean value for the number of TUNEL-labelled nuclei per unit area of lentoid, whereas 100ng/ml TNFα gave a significantly lower value. Since 100 ng/ml gave the highest value after 4 days in culture, it is probable that this apparently lower value for TNFα after 6 days in culture is due to the fact that a greater number of nuclei have already disappeared at this time, due to the higher concentration of TNFα. It follows that, since they are no longer present, they are no longer available to be labelled by the TUNEL method.

ii. Anti-TNFR1 and anti-TNFR2: The apparent difference between the effects of TNFα and the effects of agonistic antibodies to TNFRs on lentoid area, after 2 days of culture, might be explained by the fact that the experiments utilising agonistic antibodies to TNFRs were performed on cultures grown on Matrigel instead of collagen IV. However, the differentiation of cultures on Matrigel was very similar to that of cultures grown on collagen IV; for example, in terms of the morphological appearance of the cultures and δ-crystallin expression patterns. In addition, there may be subtle differences between the cellular effects of agonistic antibody binding to TNFRs and the consequences of TNFα-TNFR binding in the same cell type. For example, agonistic antibody binding to a particular TNFR sub-type may prevent binding of TNFα to the same TNFR sub-type or may be able to initiate a particular cellular response such as cytotoxicity, but not proliferation (Heller *et al.*, 1993). This might explain the different effects of TNFα and agonistic TNFR antibodies on lentoid area reported here, if agonistic antibodies are considered to have a more pronounced effect on lentoid cell denucleation than on lens epithelial cell proliferation in this system.

In the experiments described here, agonistic antibodies to TNFR1 and TNFR2 were not significantly different from each other with regard to lentoid cell denucleation, suggesting that chick TNFR1 and TNFR2 appear to each have a direct effect on this process. Therefore,

both TNFRs could be involved in mediating the denucleation of lentoid cells, since both agonistic antibodies individually promoted this process. This is despite the fact that, from the immunofluorescence data, TNFR2 appears to be much less abundant in lens fibre cells than TNFR1. Each antibody could also cause up-regulation of TNFα production (see Chapter 2), which could then bind to the TNFR sub-type not bound by the agonistic antibody. This possibility could be investigated by incubating the cultures with anti-TNFα at the same time as agonistic TNFR antibodies, since this would inhibit any effects of endogenous TNFα production. It is also possible that there is a degree of functional cross-reactivity of the agonistic anti-mouse TNFR1 antibody with chicken TNFR2 and, similarly, of anti-mouse TNFR2 with chicken TNFR1, since the specificity of these antibodies for each of the equivalent chicken TNFRs is unknown.

Finally, in view of the fact that the TNFα-related molecule identified in Western blots here is possibly membrane bound and TNFR2 is capable of promoting lentoid cell denucleation, it is interesting that transmembrane TNFα has been shown to be the prime activating ligand of TNFR2 (Grell *et al.*, 1995).

iii. Anti-TNFα: The effects of anti-TNFα on lentoid denucleation were conclusive and suggest that endogenous production of a TNFα-like factor by lens fibre cells contributes significantly towards their denucleation. After 2 days in culture, there was an approximately 7 fold reduction in the number of labelled nuclei per unit area per measurement compared to rabbit serum at the same concentration, a 2.5 fold reduction compared to preabsorbed antibody, and a 5.5 fold reduction compared to cultures in defined medium alone. After 4 days in culture, there was a 29 fold reduction compared to cultures in defined medium alone. It was difficult to gauge the effects of anti-TNFα after 4 days in culture because the effect of anti-TNFα was to cause the lens epithelial cells in the monolayer to detach from each other, resulting in disruption of the monolayer and the formation of thin, strand-like remnants of the monolayer. This effect could be due to decreased cell-cell adhesion, since TNFα has been shown to affect cell-cell adhesion via affecting actin stability in epithelial cells (Tabibzadeh, 1995).

7.4.4. Statistical methods

In the present study, the unpaired, two-tailed student's t-test was used to obtain values of statistical significance between the means of groups of experimental data obtained using image analysis. These values are presented above and in Appendix II. However, Zar (1984) has pointed out that the application of two sample t-tests to all possible samples in a multisample experiment is not generally valid, since, as the number of samples increases from two, there is an increasing probability of committing a type I error; i.e. an increasing probability of concluding that the means of the two most extreme samples are significantly different from each other, when in fact they are not.

In the present study, if the multiple tests on lens epithelial cell cultures receiving 0, 10, 50, and 100 ng/ml TNFα are considered, the number of sample means (k) is 4. Zar (1984) has presented the probability of committing a type I error dependent upon the level of significance used in the t-test and the number of sample means. For example, when $k = 4$ there is a 0.6% chance at $p = 0.001$ of committing a type I error, a 5% chance at $p = 0.01$, and a 23% chance at $p = 0.05$. Since, in the present study, the highest levels of significance obtained using multiple t-tests were often less than $p = 0.001$ (inaccuracy in the test is 0.6%), it is reasonable to trust the accuracy of the multicomparison t-tests performed here when $p < 0.001$. At levels of significance at $p = 0.05$, the tests are less conclusive and interpretation of the results of these tests should be more cautious.

Zar (1984) suggests that greater accuracy can be obtained, when analysing the degree of significance between multiple samples, by using a multiple comparison test. First, analysis of variance (ANOVA) should be used on the data to reject a multisample hypothesis of equal means; i.e. to show that the means obtained in the samples are different from each other. This should then be followed by a multiple comparison test. There are several commonly used multiple comparison tests; for example the Tukey test and the Newman-Keuls test. With regard to the data presented here and in Appendix II, it would be most useful to perform the Tukey test as recommended by Zar (1984).

were due to a direct influence on lens fibre denucleation or due to an indirect effect on overall entoid differentiation. An excellent marker for lens fibre differentiation in the chick is δ-crystallin, since this is massively up-regulated in chick lens fibre cells (Piatigorsky, 1984). It is feasible that TNFα could be involved in crystallin synthesis, since crystallin belongs to the same family as the heat shock proteins (HSPs; see Chapter 6) and TNFα is able to induce HSPs during situations producing cellular stress (see Chapter 2). However, in the quantitative Western blotting study performed here, there was no apparent effect of TNFα or anti-TNFα on δ-crystallin deposition in lens epithelial cell cultures, since the intensity of the approximately 45-50kDa δ-crystallin band observed is similar in each case (Fig. 7-11), implying that the effects of TNFα on lens fibre denucleation are specific for that process. The lower molecular weight band at approximately 18kDa could be a physiological breakdown product of δ-crystallin (D. Beebe, personal communication).

7.4.6. Bcl-2, bax, and ICE expression

It is important to gain an understanding of the nature of factors that may regulate the effects of TNFα-like factors during development. In the present study, immunoreactivity for bcl-2, a known inhibitor of TNFα-mediated cell death (Hennet *et al.*, 1993) was identified in the lens epithelium and in cortical lens fibres. Thus, bcl-2 may be able to protect these cells from TNFα-mediated death and denucleation. Bax is related to bcl-2 and heterodimerises with it to inactivate it, thus promoting cell death (Reed, 1994). In the present study, the expression of bax in central lens fibres could be indicative of a role for it in lens fibre cell denucleation by inhibiting bcl-2 function in central lens fibres.

Finally, ICE is a protease that is homologous to the *C. elegans* gene *ced-3*, which has a role in programmed cell death (Sanders and Wride, 1995). It appears to be expressed in lens epithelial cells in which apoptosis is occurring (Ishizaki *et al.*, 1993) and its expression in lens fibre cell nuclei and in a punctate pattern in central lens fibres could suggest a role for it in lens

fibre cell denucleation mediated by TNFα-like factors, since the cowpox virus *crmA* inhibits ICE and Fas and TNFα-mediated apoptosis (Tewari and Dixit, 1995; Enari *et al.*, 1995)

7.4.7. Final comments

Interestingly, another pro-inflammatory lymphokine has recently been identified in the developing eye lens, macrophage migration inhibitory factor (MIF; Wistow *et al.*, 1993). MIF, like TNFα, has been shown to be a pivotal mediator in endotoxic shock (reviewed by Bucala, 1994) and the two may act directly to augment each other's production in a reciprocal pro-inflammatory loop. Wistow *et al.* (1993) showed that the expression of MIF in the lens is strongly correlated with lens fibre cell differentiated and suggested that MIF could have autocrine/paracrine roles in the lens separate from its role in the immune system. It is suggested here that that TNFα-like factors may fall into the same category, since they appear to have a role in lens fibre cell denucleation. In addition, the expression of TNFα-like activity in the developing lens could have important clinical implications; for example, in ocular inflammation, as pointed out by Wistow *et al.*, for MIF.

In conclusion, TNFα-like proteins and TNFRs have been identified in differentiating lens cells both *in vivo* and *in vitro*. It has been demonstrated that the addition of exogenous TNFα and agonistic antibodies to TNFRs to cultures enhances the rate of nuclear degeneration in lentoids and the addition of polyclonal neutralising antibody to TNFα inhibits this process. Thus, proteins with TNFα-like activity could have an important role in lens fibre cell denucleation during chick embryo development. In addition, a greater appreciation of the factors that regulate this process could lead to advances in the understanding of factors that contribute towards certain congenital cataracts; e.g. during rubella virus infection of pregnant mothers (see Chapter 6), and in the understanding of the factors that contribute towards ocular inflammation.

TABLE 7-1

Summary of lens culture data: nuclei per area

2 day lens culture experiments											
Test type	*TNF 0	TNF 10	TNF 50	TNF 100	Anti-TNF	PA	RS	Test type	TNF 0	TNFR1	TNFR2
Mean	3.27	4.05	4.68	6.70	0.58	1.43	4.02	Mean	3.85	6.25	7.33
Max	7.43	6.53	9.31	10.29	1.27	2.09	7.54	Max	9.07	10.27	12.25
Min	1.34	2.52	2.84	2.89	0.10	0.00	1.22	Min	1.43	2.83	3.75
n	31	20	35	22	20	10	11	n	19	19	19
St Dev	1.41	1.06	1.53	1.93	0.32	0.59	2.20	St Dev	1.95	2.11	2.01

4 day lens culture					
Test type	TNF 0	TNF 10	TNF 50	TNF 100	Anti-TNF
Mean	3.48	3.96	6.46	5.20	0.12
Max	7.58	8.85	14.75	10.08	0.33
Min	1.67	1.41	2.26	2.82	0.00
n	24	44	35	35	11
St Dev	1.48	1.47	2.84	1.93	0.15

6 day lens culture				
Test type	TNF 0	TNF 10	TNF 50	TNF 100
Mean	4.92	5.07	7.43	3.22
Max	14.50	9.65	10.53	7.65
Min	0.90	1.78	1.13	0.00
n	32	28	21	21
St Dev	2.65	1.87	2.93	1.88

Notes: All TNF concentrations in ng/ml

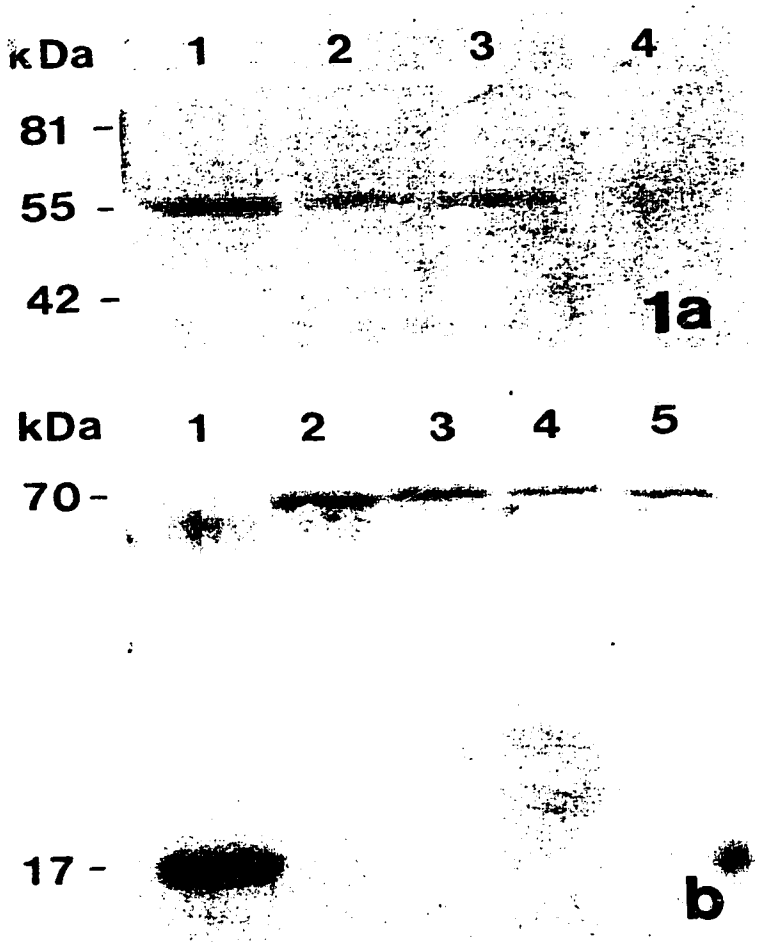

Figure 7-1. Western blot analysis of lenses from ED 6-15 homogenised in protease inhibitor buffer. (a). Western blot using polyclonal antibodies to mouse TNFR1. Lane 1: ED 6 lens; Lane 2: ED 8 lens; Lane 3: ED 11 lens; Lane 4: ED 15 lens. **(b).** Western blot using polyclonal antibodies to mouse TNFα. Lane 1: recombinant mouse TNFα probed with polyclonal anti-mouse TNFα; Lane 2: ED 6 lens; Lane 3: ED 8 lens; Lane 4: ED 11 lens; Lane 5: ED 15 lens.

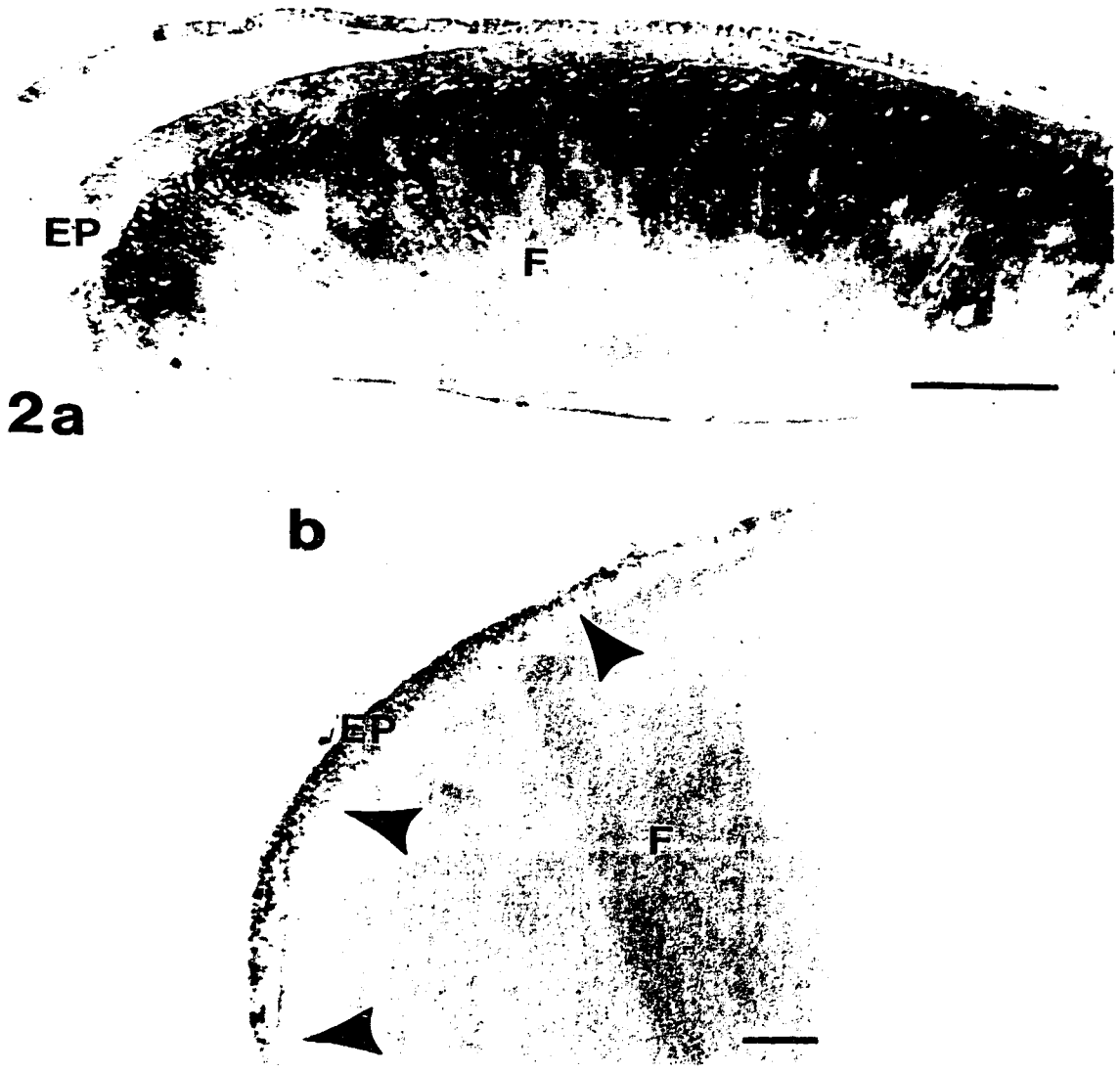

Figure 7-2. Sections of leases stained with polyclonal antibodies to mouse TNFα. (a). ED 5 lens. Lens fibres (F) are immunoreactive. **(b).** ED 11 lens. Lens fibres have lost their immunorcactivity and staining becomes localised to the lens epithelium (EP). Bars = 50 μm

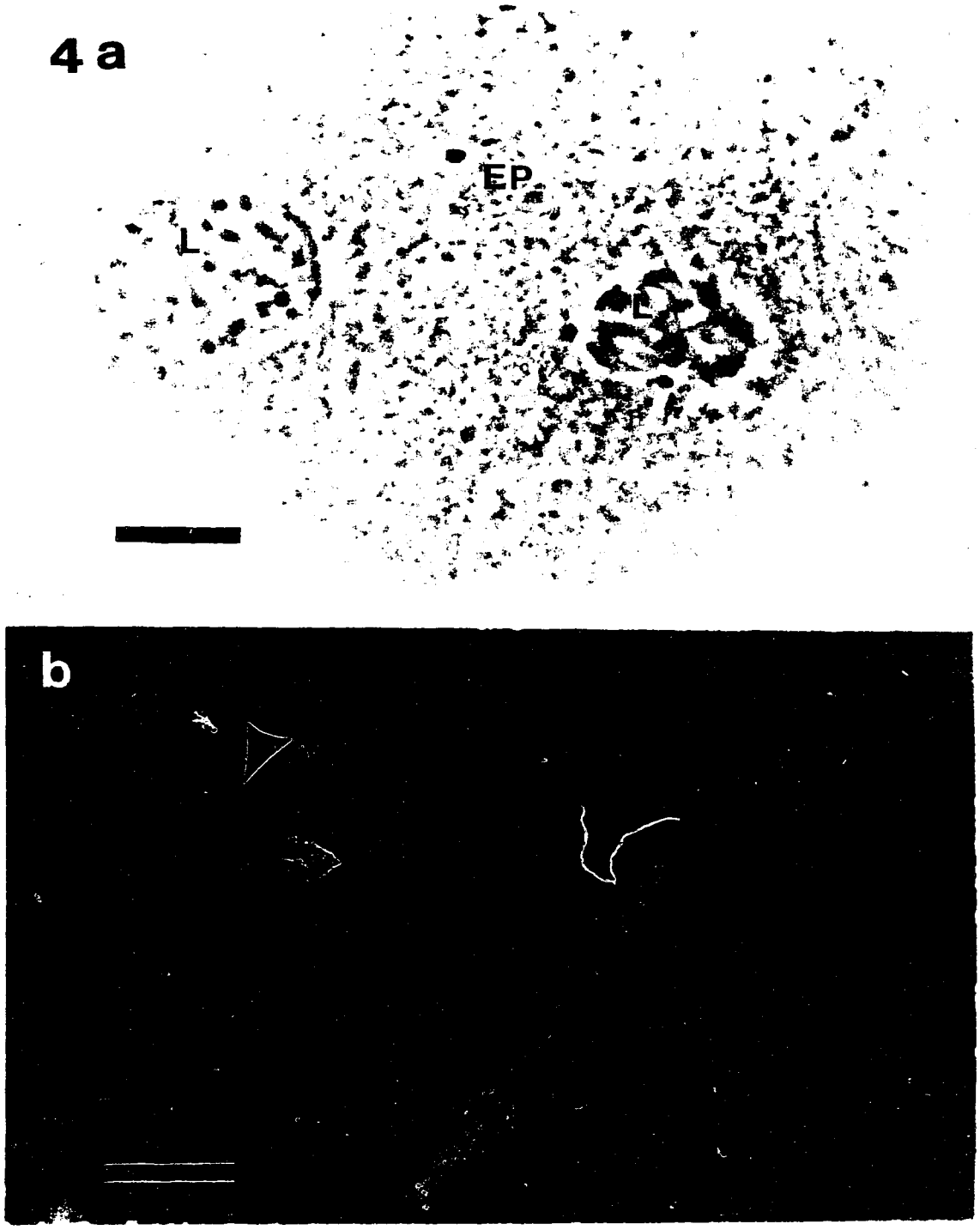

Figure 7-4. Lens epithelial cells differentiating in culture and exhibiting TNFα immunofluorescence. **(a).** Phase contrast micrograph of lens epithelial cell monolayer (EP) in culture, and differentiation of lens epithelial cells into clumps of lens fibre-like cells called lentoids (L). **(b).** TNFα immunofluorescence at periphery of lentoids (arrowhead) in the same culture as in (a). Bars = 50 μm.

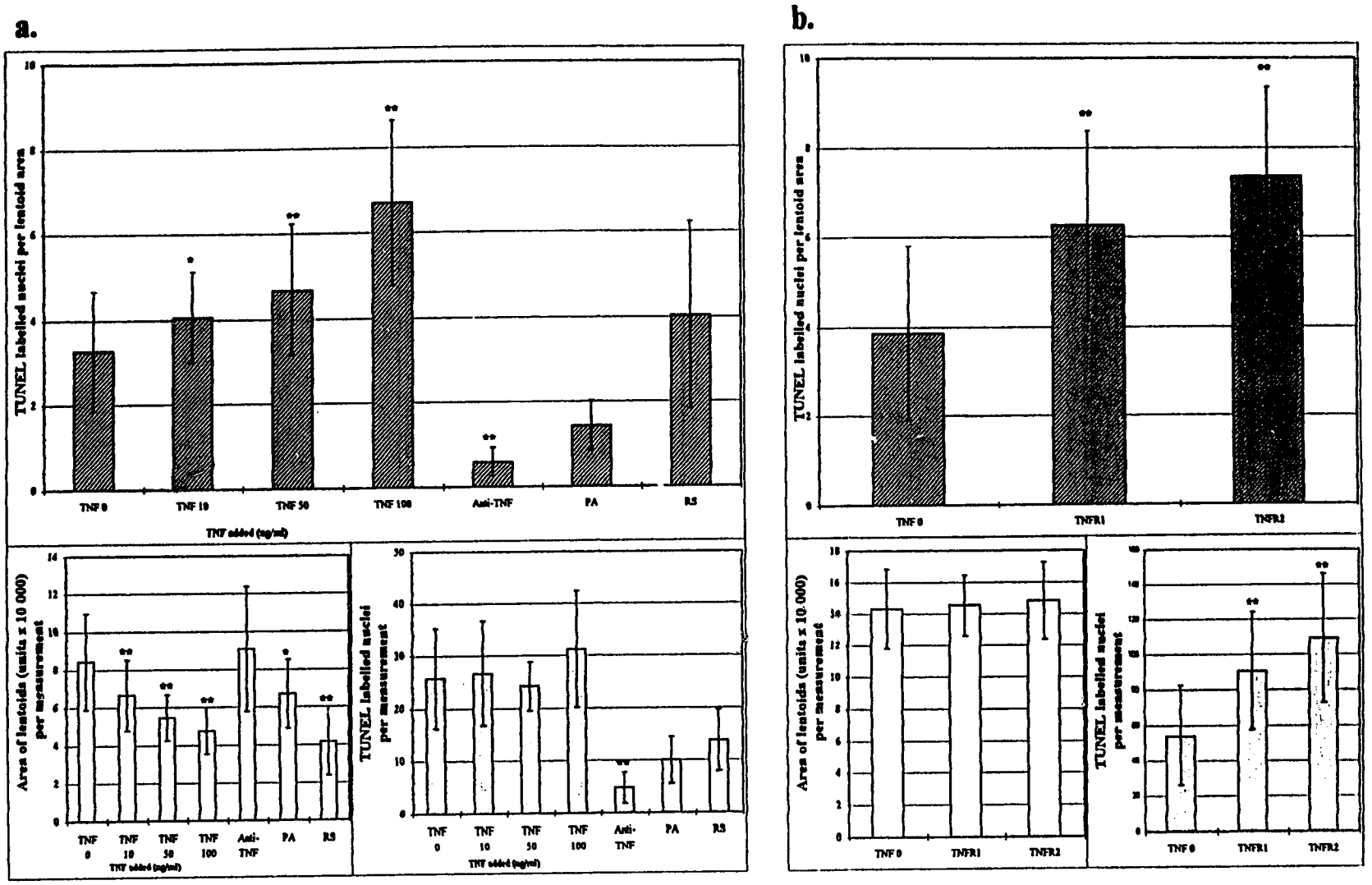

Figure 7-7. Effect of TNFα, anti-TNFα, anti-TNFR1 and anti-TNFR2 on the degeneration of lentoid nuclei in chick embryo lens epithelial cell cultures after 2 days. (a). Effect of TNFα and anti-TNFα on the degeneration of lentoid nuclei in chick embryo lens epithelial cell cultures after 2 days. **(b).** Effect of agonistic antibodies to TNFR1 and TNFR2 on the degeneration of lentoid nuclei in chick embryo lens epithelial cell cultures after 2 days.

* $p < 0.05$; ** $p < 0.01$ compared to control (see text); Error bars ± SD. PA, preabsorption control; RS, rabbit serum control

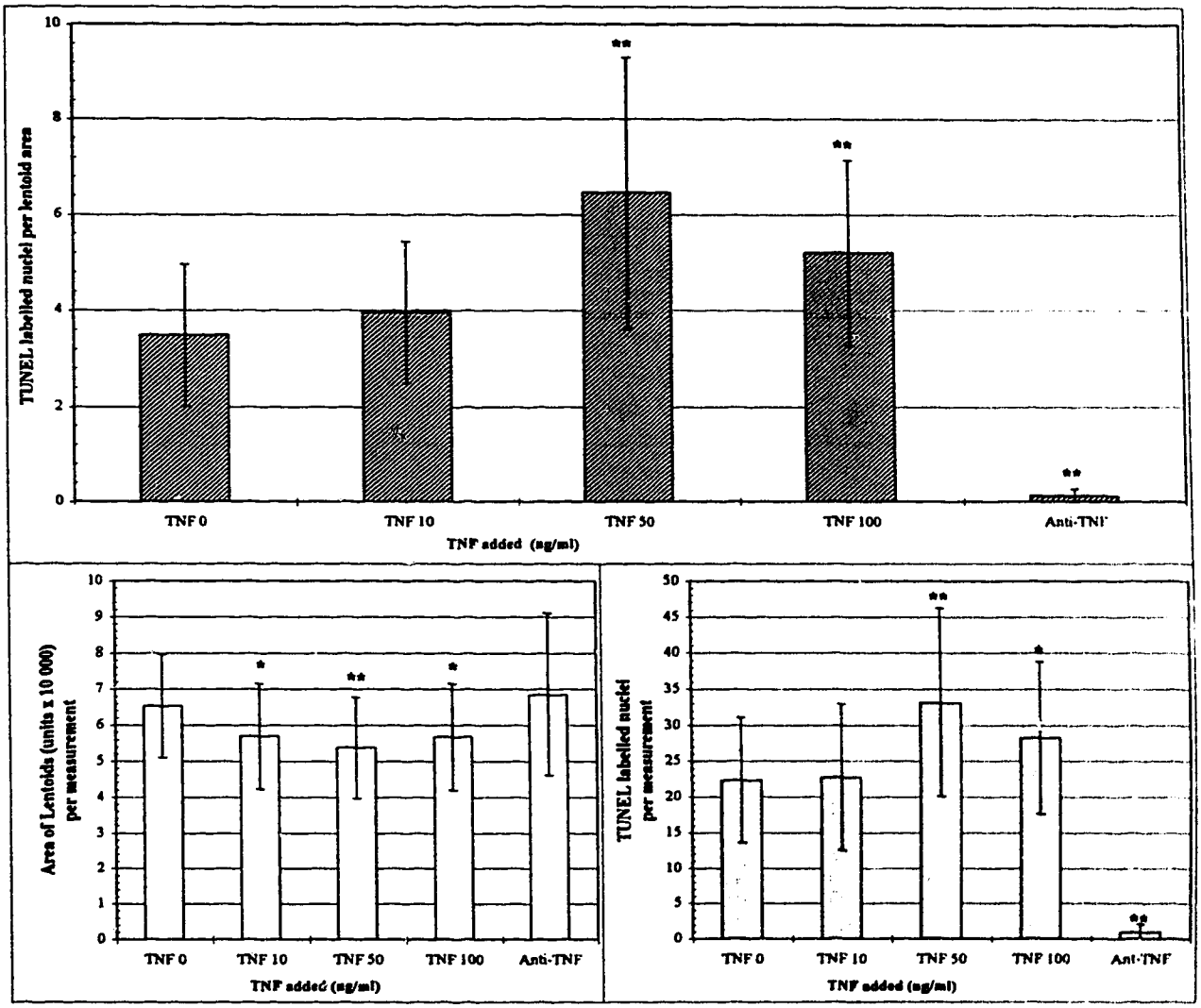

Figure 7-8. Effect of TNFα and anti-TNFα on the degeneration of lentoid nuclei in chick embryo lens epithelial cell cultures after 4 days. * $p < 0.05$; ** $p < 0.01$ compared to control (see text); Error bars ± SD. PA, preabsorption control; RS, rabbit serum control

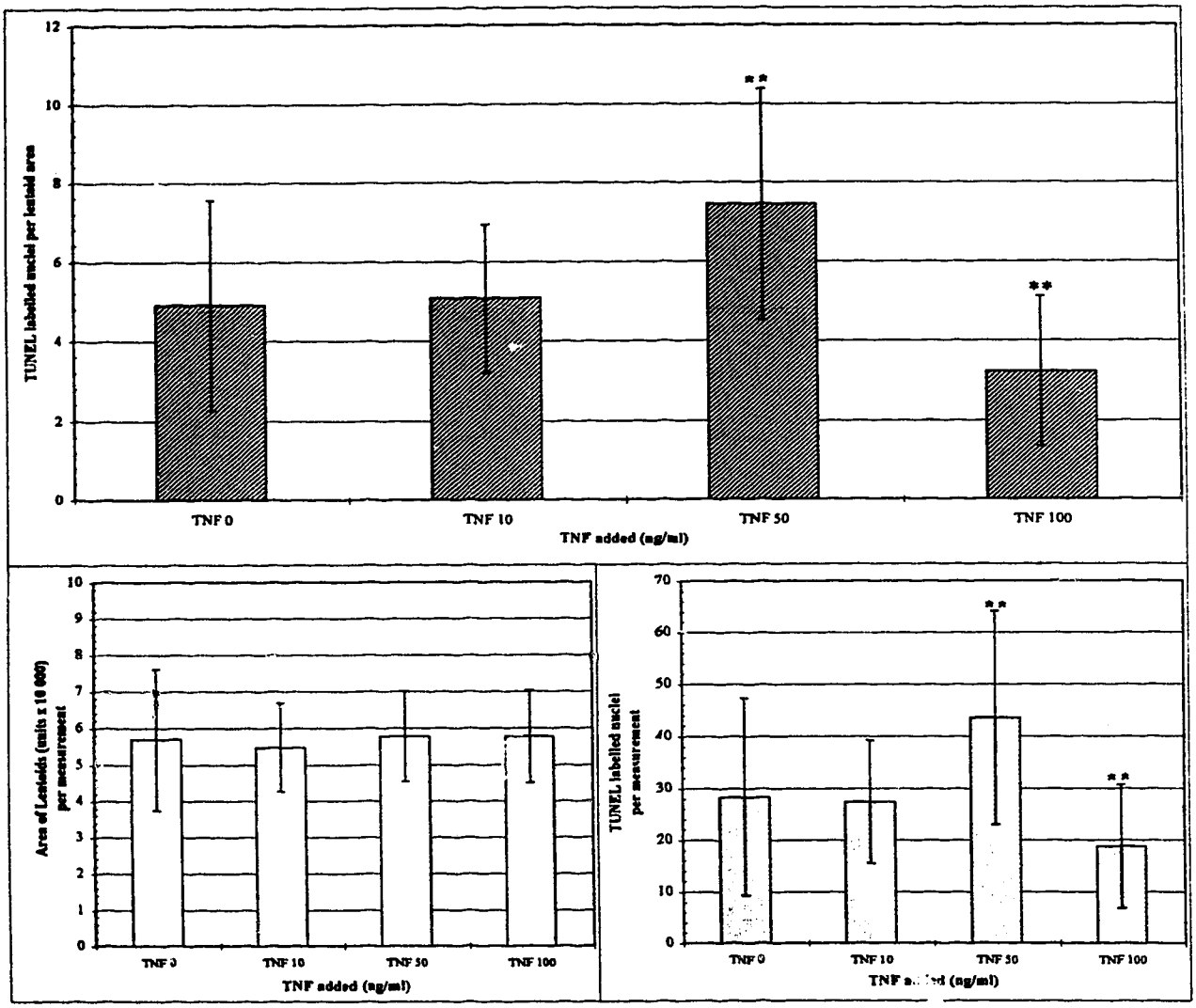

Figure 7-9. Effect of TNFα on the degeneration of lentoid nuclei in chick embryo lens epithelial cell cultures after 6 days. * $p < 0.05$; ** $p < 0.01$, compared to control (see text); Error bars ± SD. PA, preabsorption control; RS, rabbit serum control

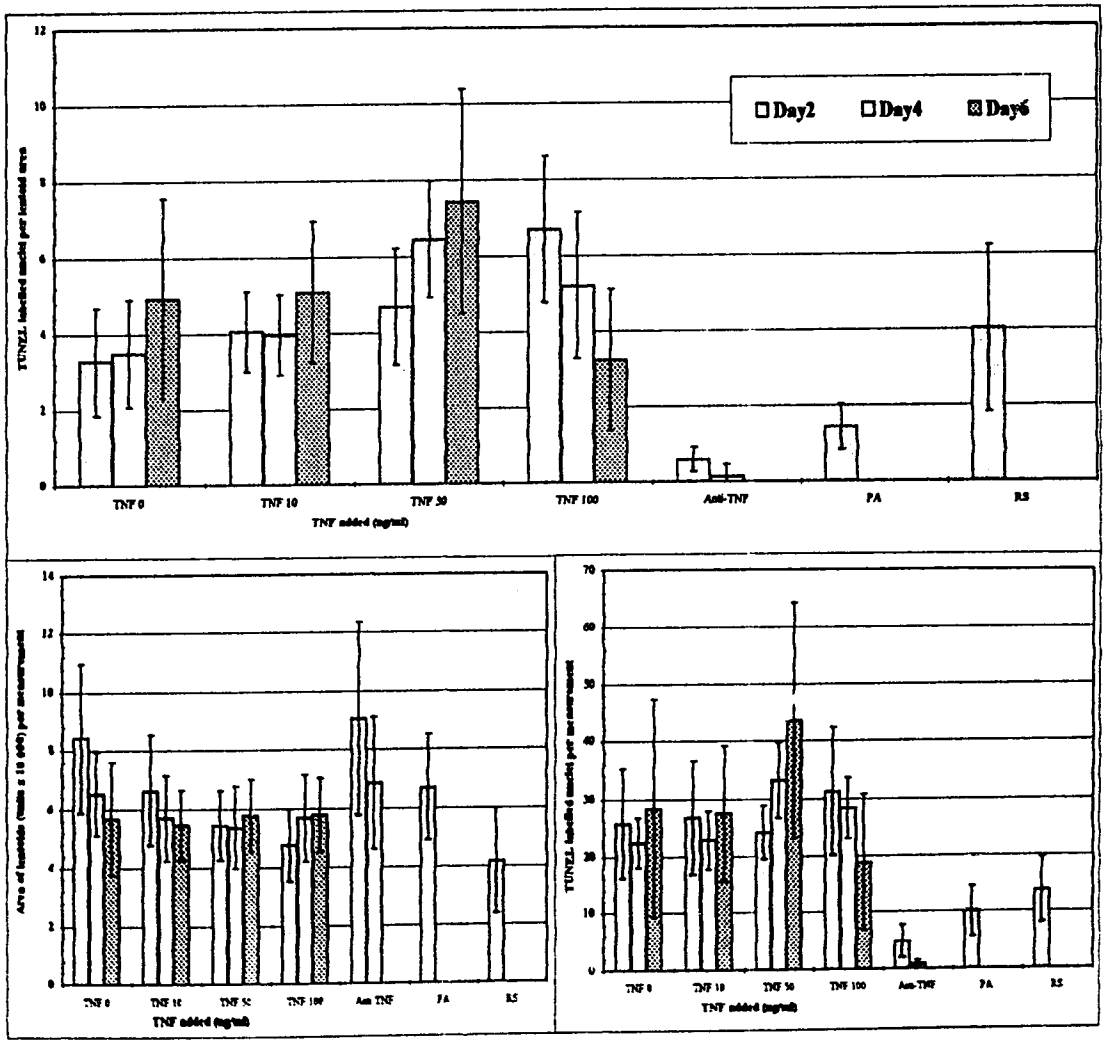

Figure 7-10. Summary of the effect of TNFα and anti-TNFα on the degeneration of lentoid nuclei in chick embryo lens epithelial cell cultures through 2-6 days. Error bars ± SD. PA, preabsorption control; RS, rabbit serum control

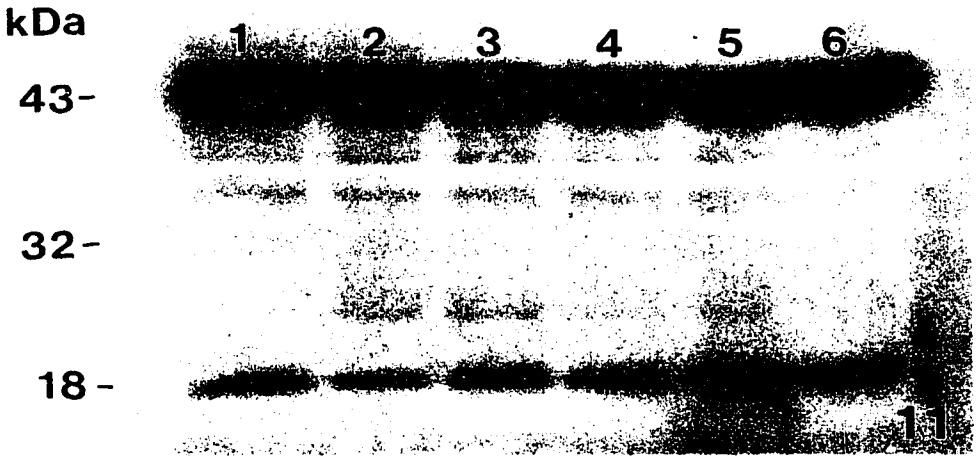

Figure 7-11. Western blot analysis of δ-crystallin expression in lens cultures. Western blot of 3 day cultures of lens epithelial cells (grown on matrigel), under different conditions, probed with polyclonal antibodies to chicken δ-crystallin. Lane 1: cultures incubated with polyclonal anti-mouse TNFα; Lane 2: cultures incubated with polyclonal anti-mouse TNFα preabsorbed with mouse TNFα; Lane 3: cultures incubated with 100 ng/ml TNFα; Lane 4: cultures incubated with 50 ng/ml TNFα; Lane 5: cultures incubated with 10 ng/ml; Lane 6: control cultures, incubated with defined medium alone.

Figure 7-12. Confocal microscope image of section of ED 15 lens stained with polyclonal antibody to mouse/human bcl-2. Bcl-2 immunoreactivity is present in the lens epithelium (EP) in the annular pad region (arrows), but immunoreactivity is less intense in lens fibres (F). Bar = 50 μm.

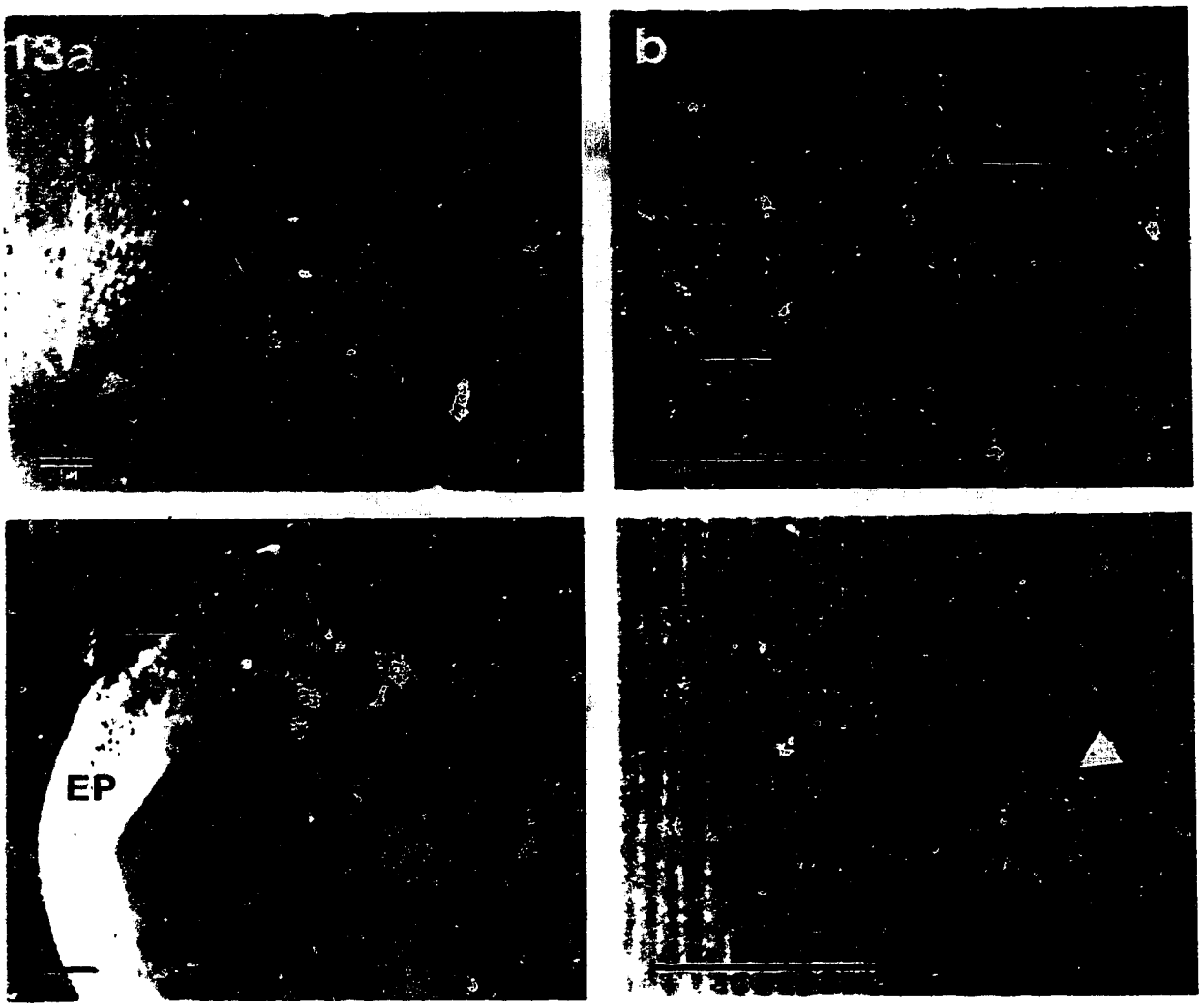

Figure 7-13. Sections of ED 8 and ED 15 lenses stained with polyclonal antibody to mouse/human Bax. (a). ED 8 lens section. Bax is expressed in the central lens fibre cells (arrowheads). (b). Higher magnification of (a). Bax immunoreactivity is associated with the central lens fibre cell membranes (lens fibres are seen in cross section), but not with lens fibre nuclei (arrow). (c). ED15 lens section. Bax is expressed mainly in the lens epithelium (EP). (d). Higher magnification of (C). Bax immunoreactivity is associated with central lens fibre cell membranes, but not with nuclei (arrow; lens fibres are seen in horizontal section). Bars = 50 μm.

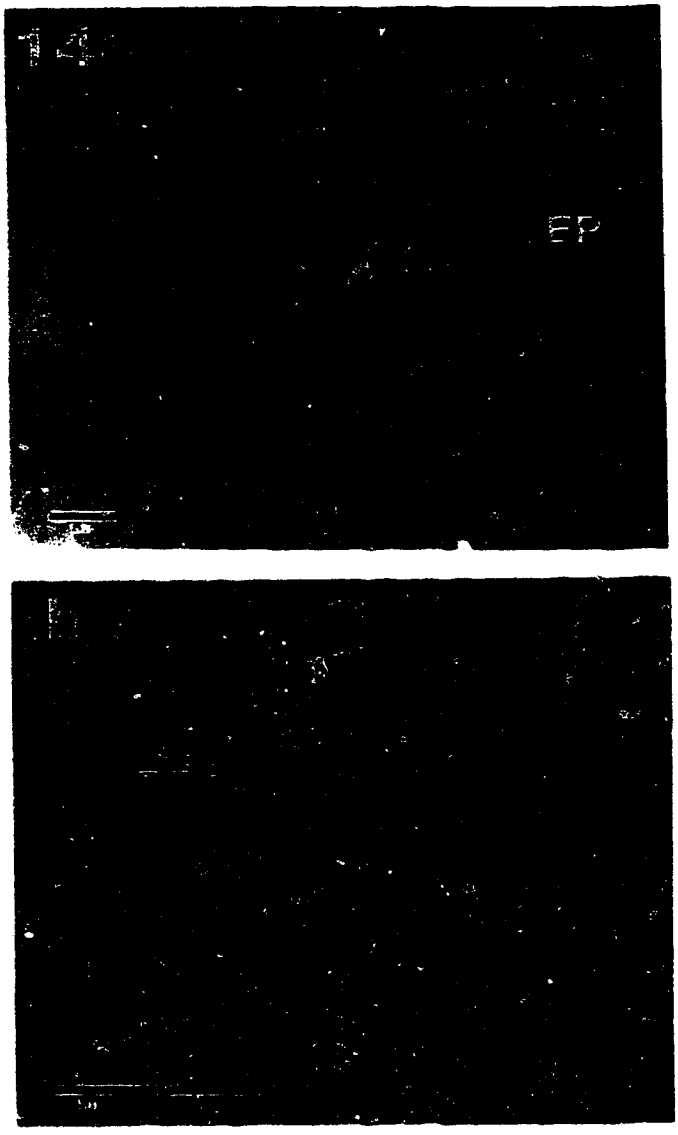

Figure 7-14. Section of ED 8 lens stained with polyclonal antibody to mouse/human ICE. (a). ICE immunoreactivity is associated with the lens epithelium (EP), lens fibre cell nuclei (arrow), and with the central lens fibre cells. **(b).** Higher power of (a). ICE is expressed in a punctate pattern (arrows) in the central lens fibre cells. Bars = 50 μm.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS[1]

8.1. Overview

TNFα is a highly pleiotropic molecule with a plethora of important roles in many aspects of physiology and pathophysiology (see Chapter 2). The studies performed here have provided evidence that avian TNFα-like factors may have important roles to play in embryonic development in the chick, since TNFα-related molecules of approximately 17kDa, 50kDa, 70kDa, and 120kDa have been localised to specific tissues at various stages of chick embryo development (Chapters 4 and 5). The development of a hypothesis proposing specific roles for TNFα-like molecules in several aspects of embryonic development (Chapter 3) proved particularly useful. This hypothesis included the proposal that TNFα may have a role in programmed cell death during development, since it is capable of inducing apoptosis in sensitive cells (Chapter 2). Indeed, chick embryo homogenate was shown to contain TNFα-like cytotoxic activity in a TNFα bioassay and these TNFα-like factors were shown to be expressed in a number of tissues undergoing programmed cell death as identified using the TUNEL technique, which detects cells undergoing DNA fragmentation (see Chapter 5). Lens development provides a particularly useful model system for the study of a number of important processes occurring during embryonic development (see Chapter 6). A 70kDa TNFα-related protein was identified at various stages of chick embryo lens development, immunoreactivity for TNFRs was also present, and recombinant mouse TNFα was subsequently shown to have a significant effect on lens fibre cell denucleation when added to chick embryo lens epithelial cells differentiating in culture (Chapter 7).

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[1] *Parts of this Chapter have been published. Wride M A and Sanders E J. (1995). Potential roles for tumour necrosis factor α during embryonic development. Anat. Embryol. 191:1-10.*

8.2. Potential means of regulation of TNFα-mediated effects during development

Early in the study of TNFα, there was a hope that it would be effective in cancer therapy because of its ability to cause tumour regression in animal models (see Chapter 2). However, TNFα is not as effective in humans as was initially expected and the doses required are highly toxic to the patient. It follows, therefore, that the expression, during embryonic development, of a molecule that is as potentially lethal as TNFα should be tightly regulated and controlled. The nature of this control is not understood, but a cell surface association of TNFα (see Chapter 4) on cells during development may provide a means of localising its effects, while the presence or absence of soluble TNFRs (sTNFRs) in the ECM (Higuchi and Aggarwal 1992; see Chapter 2), or the sequestration of TNFα in the ECM, through its binding to fibronectin or laminin (Alon *et al.*, 1994) may provide additional means of regulation. Furthermore, it is known that expression of the oncoprotein bcl-2 is developmentally regulated and can prevent apoptosis of cells in which it is expressed (Eguchi *et al.*, 1992) and that it may prevent TNFα-induced apoptosis (Hennet *et al.*, 1993). Immunoreactivity for bcl-2, and the related molecule bax, has been observed here in the developing lens with a distribution that may reflect potential roles for it in the modulation of TNFα-mediated effects on lens fibre cell denucleation (see Chapter 7). In addition, bcl-2 and bax are expressed during gastrulation stages in the chick embryo and may be involved in the regulation of TNFα-mediated effects on programmed cell death at these stages (Sanders *et al.*, 1996) Thus, the cytotoxic effects of developmental TNFα expression may be regulated by members of the *bcl-2* oncogene family.

8.3. TNFα and reaper

The most suggestive data in support of the proposal put forward here, that TNFα-like factors may have important roles to play during embryonic development, is that there is a significant degree of homology between the *Drosophila* cell death-promoting protein reaper, the death domains of Fas and TNFR1, and proteins involved in axonal guidance (Golstein *et al.*, 1995; Hofmann and Tschopp, 1995). Reaper is a protein central to the control of

programmed cell death during *Drosophila* embryogenesis and reaper mRNA is expressed in cells destined to undergo apoptosis (White *et al.*, 1994; White and Steller, 1995; Sanders and Wride, 1995). In addition, a number of the TRAFs (Chapter 2), including TRADD, FADD, and RIP, are also homologous to reaper and the death domains of TNFR1 and Fas, particularly in their middle one third (Cleveland and Ihle, 1995). These observations suggest that cell death signalling pathways, which contain TNFR1- and TRAF-related proteins, are important during development and have been conserved during evolution (Golstein *et al.*, 1995). Thus, an involvement for TNFα-like factors and TNFRs in vertebrate programmed cell death is a reasonable proposal.

8.4. TNFα-inhibitors and TNF receptor knockouts

Clues to the potential roles of TNF ligand and receptor family members in development may also be provided by studies in which members of this family are deleted or in which inhibitors of TNF ligand function are overexpressed (see Chapter 2). The results of these studies have suggested that TNFα and TNFRs may be dispensable during embryonic development. However, the evidence from transgenic studies is not conclusive enough to rule out a role for TNFα, or other TNFα-related molecules, in development for a number of reasons. For example, lack of a phenotypic effect associated with the deletion of both TNFRs simultaneously does not preclude the possibility that TNFα is capable of interacting with a TNFR distinct from TNFR1 and TNFR2. Indeed, Schwalb *et al.* (1993) have identified an alternative TNFR sub-type that is specific for TNFα and does not bind LTα. Thus, TNFα may have developmental effects that are not mediated through TNFR1 or TNFR2. Alternatively, TNFα may have its own signalling activity independent of TNFRs, either through a direct signalling capability mediated by its membrane bound form, or, since it has been shown that the TNFα trimer can form ion channels that mediate cellular responses (Kagan *et al.*, 1992; 1993; see Chapter 2), the TNFα trimer may induce cell signalling directly through ion channel formation. It is also likely that the full complement of TNF ligand and receptor molecules is

yet to be identified and this is apparent in the present results, since a number of higher molecular weight avian TNFα-like factors have been revealed during chick embryo development, and TNFα-related molecules of similar sizes have been identified during mouse lung development (Jaskoll *et al.*, 1994). Indeed, as part of the Human Genome Project, tens of thousands of cDNAs have been cloned and sequenced and several of these human cDNAs share high homology with human TNFα and LTα (M. Marino, personal communication). Thus, there appear to be other TNFα-related molecules in humans and, therefore homologues of these probably also exist in mice. Thus, additional forms of TNFα and their receptors may be able to compensate for lack of TNFα or TNFR1 and/or TNFR2 in transgenic mice. In addition, these forms of TNFα may have very specific, localised effects during embryonic development, which are not as susceptible to inhibition by injection of antibodies or the injection or overexpression of TNFα inhibitory constructs (Peppel *et al.*, 1993). Indeed, these authors realised this and stated that they could not rule out the possibility that a fraction of locally produced TNFα, LTα, or LTβ in these mice might bind to TNFRs on neighbouring cells during development, particularly if TNFα inhibitor expression in a particular tissue was low. In this regard, it is perhaps useful to think about the possibility of there being two pools of TNFα in the body, a pool of soluble TNFα and a pool of TNFα with a very restricted and localised pattern of expression, resulting from sequestration in the ECM or as a result of being membrane bound. This latter pool of TNFα may be less sensitive to the injection of neutralising antibodies to TNFα or to the expression of inhibitory constructs than the soluble pool. It is also possible that the sensitivity of cells to TNFα might be increased by the presence of inhibitors (perhaps by upregulation of TNFRs at the cell surface) or the production of TNFα, LTα, or LTβ might be increased by the presence of the inhibitor. In addition, it has been demonstrated that injection of human volunteers with LPS resulted in elevated TNFα levels, which correlated with the onset of septic shock symptoms, such as fever (Kuhns *et al.*, 1995). Accompanying these events, there was a 12 fold increase in the level of sTNFRs. Thus, a 12 fold excess of sTNFR appears to be insufficient to quench all TNFα activity and,

even though a much higher activity of inhibitor was achieved by Peppel *et al.* (1993) in their transgenic mice, it is still possible that not all TNFα activity was removed in these mice. It may be significant that gene knockout experiments on other proteins with a putative involvement in development have also given negative results, although this does not necessarily suggest that their expression is superfluous; i.e. occurring in tissues in which they have no function, due to lack of receptors (reviewed by Erickson, 1993).

8.5. Characterisation of avian TNFα-like factors

One major observation in this thesis is that there are a number of forms of TNFα-like proteins expressed during chick embryo development. The exact nature of these molecules remains undetermined and, indeed, in the chick, TNFα has not yet been cloned and sequenced (see Chapter 2). It will, therefore, be important to clone and sequence the genes that encode for the TNFα-cross-reactive proteins identified here. This could involve initial characterisation of these proteins by microsequenceing and the information gained could be used to subsequently create degenerate oligonucleotide primers that could be used for RT-PCR amplification of avian TNFα-related cDNAs. These cDNAs could then be used to screen a chick embryo cDNA library to obtain full length cDNAs of the chick TNFα-related proteins. A similar method to that employed by Wistow *et al.* (1993), to clone chicken lens MIF, may be useful for cloning the chicken TNFα-related factor expressed in the developing lens. In this case, a chick embryo lens cDNA library would be screened. It is possible that chick embryo TNFα-like factors, cloned and sequenced in this way, might be similar to members of the TNF ligand family already identified in mice and humans (see Chapter 2).

8.6. Implications of embryonic TNFα expression for cancer treatment

Since TNFα has an important involvement in a number of disease states (see Chapter 2), a knowledge of the functions of TNFα during development may add to the understanding of a number of pathophysiological processes in which it is involved.

For example, as stated in Chapter 2, William Coley was able to make use of the effects of TNFα (although he did not realise this at the time) following erysipelas infections in his treatment of patients with cancer, particularly those with sarcomas. The reason why sarcomas should have responded preferentially to Coley's toxins remains unanswered. Sarcomas are malignancies derived from mesodermal tissues and it has been suggested that these types of tumours have a higher probability of being immunogenic; i.e. susceptible to effects mediated by cytokines, such as TNFα, produced by the immune system (Wiemann and Starnes, 1994). Thus, it has been suggested by Wiemann and Starnes (1994) that:

> "...the possibility, therefore, exists that if we understood more precisely the nature and role of these antigens and cytokines in embryonic development, we might also have a better concept of why it was that tumours of certain embryonic origin responded preferentially to Coley's therapy. It may well be that the treatment that Coley developed was taking advantage of a system that was designed for other purposes; i.e. control over embryonic development and differentiation, rather than immune surveillance of cancer."

Thus, it is certainly worth pursuing this idea and, indeed, a new less toxic form of Coley's toxins has been developed, which may be effective in cancer treatment (Mizuno and Soma, 1992).

8.7. Thalidomide defects

A role for TNFα in development may help to explain the teratogenic effects of thalidomide. Thalidomide was administered to women in the first trimester of pregnancy, as a treatment for nausea, in the late 1950s and early 1960s. However, as is well known, it often resulted in severe defects in the foetuses (reviewed by Mellin and Katzenstein, 1962). Recently, it has come to light that thalidomide has the ability to selectively inhibit TNFα production by stimulated human monocytes (Sampaio *et al.*, 1991) by specifically inducing

degradation of TNFα mRNA (Moreira *et al.*, 1993). Thus, it is possible that thalidomide could have its teratogenic effect by inhibiting developmentally regulated TNFα expression. A hypothesis concerning the mechanism for thalidomide induced teratogenicity has been put forward by Lash and Saxén (1971; 1972). These authors noticed that thalidomide inhibited induction of cartilage, by the mesonephros, in cultured limb tissue and that radioactive thalidomide appeared to bind specifically to the human mesonephros. Interestingly, TNFα immunoreactivity has been observed here in the chick embryo mesonephros (see Chapter 5). Thus, it is possible that the effects of thalidomide on limb bud development could perhaps be due to inhibition of TNFα expression in the mesonephros, suggesting that TNFα expression in the mesonephros may have a role as one of the signals mediating limb bud outgrowth.

An alternative explanation for the effects of thalidomide, involving effects on TNFα expression, could be related to the observation that thalidomide inhibits blood vessel growth in the developing limb bud (D'Amato *et al.*, 1994). Since TNFα is a known promoter of angiogenesis (Nakatani *et al.*, 1994) and is expressed in embryonic limb bud mesenchyme (see Chapter 4), it is possible that thalidomide could inhibit TNFα-mediated promotion of angiogenesis in the developing limb. However, it is also important to realise that thalidomide may have multiple effects in addition to those that it has on TNFα. For example, it has been proposed that down-regulation of adhesion receptors on the cells of primate embryos may be a mechanism for the teratogenic effect of thalidomide (Nogueira *et al.*, 1994; Neubert *et al.*, 1995). Future studies directed at elucidating the functions of TNFα during development could involve the use of thalidomide or other drugs that inhibit TNFα expression, such as pentoxifylline (Zabel *et al.*, 1993).

8.8. Lens fibre denucleation, TNFα, viruses, and cataract

A particularly interesting observation was described in Chapter 7, that a TNFα-like factor appears to have an involvement in the pathway of lens fibre cell denucleation. As mentioned in Chapter 6, overexpression of the E6 oncoprotein in the developing mouse lens,

under the influence of the αA-crystallin promoter, resulted in a lack of lens fibre cell denucleation. Different virus families are characterised by the different mechanisms that they employ to evade the immune system and exposure of viruses to directly antiviral cytokines, such as TNFα, has exerted a particularly strong selective pressure on viruses and led to the development of these viral immune evasion strategies (Lidbury, 1994). Indeed, cowpox virus contains two copies of an early gene encoding a protein, CrmB, which has homology to sTNFR2 (Hu *et al.*, 1994). This gene is related to the T2 protein from the myxoma virus (Schreiber and McFadden 1994), which encodes a homologue of sTNFR2 (Smith *et al.*, 1994). In addition, Epstein Barr virus can inhibit TNFα transcription (Gosselin *et al.*, 1991). It was noted in Chapter 6 that rubella virus infection of pregnant mothers can result in congenital cataract in the foetus. It is suggested here that viral infection may result in inhibition of the naturally occurring process of lens fibre denucleation in the lens through interference with the natural process of TNFα-mediated lens fibre denucleation during embryonic development.

Further experiments should be performed to investigate an involvement for TNFα in lens fibre denucleation and the hypothesis that viral proteins can inhibit TNFα-mediated lens fibre denucleation. These experiments could involve the creation of transgenic mice overexpressing TNFα inhibitors in the lens (such as that developed by Peppel *et al.*, 1993) or of viral proteins, under the influence of the αA-crystallin promoter. TUNEL analysis could then be carried out on lens sections from these mice to investigate if there are effects on lens fibre denucleation. Transgenic mice that overexpress TNFα in the lens may also provide insights into the role of TNFα in lens development and denucleation of lens fibres in these mice would be expected to occur at an advanced rate compared to controls.

8.9. Future studies

There are a number of developing tissues that express TNFα-like proteins in the chick embryo that deserve further attention with regard to the potential roles for TNFα within them.

Assessment of potential roles for TNFα during development will involve the use of both *in vitro* and *in vivo* approaches, examples of which are outlined below.

8.9.1. TNFα in the nervous system

The relationship between the expression of TNFα and programmed cell death in various parts of the nervous system needs to be addressed further and more thoroughly in future studies in the light of the observations presented in Chapters 4 and 5 of this thesis. The spinal motoneurons are undergoing cell death at the stages examined here and there is some faint TNFα immunoreactivity in the ventral horns of the neural tube during this time (see Chapters 4 and 5). However, other regions that do not exhibit TNFα immunoreactivity clearly contain TUNEL labelled (dying) cells; for example, the dorsal root ganglia. Furthermore, there are regions of the nervous system in which TNFα immunoreactivity is intense, but in which there is no TUNEL-reactivity; for example, in the marginal zone.

In the case of areas of cell death in which TNFα is not expressed, it is clear (as already stated in Chapter 5) that TNFα, if involved at all in programmed cell death in the nervous system, will most probably *not* be involved in *all* examples of this phenomenon. There are likely to be many mechanisms of programmed cell death in embryos in general and this is also true of the mechanisms employed in the nervous system. Indeed, Martin Raff (Raff, 1992; Raff *et al.*, 1993) has suggested that cells in the developing nervous system may exhibit a trait which appears to be common to many cell types, with regard to cell death, and that is that programmed cell death appears to occur by default unless it is suppressed by signals from other cells. Indeed, the nervous system provides one of the classic examples of this phenomenon, since it has been known for a number of years that cell survival in the developing nervous system is dependent upon neurotrophins, such as NGF, which promote cell survival and this has been called the neurotrophic theory (Raff *et al.*, 1993). In considering programmed cell death in the nervous system, it has to be taken into account, in the light of the results presented here, that there may also be factors present in the environment of developing nerves, such as

TNFα, which promote rather than inhibit cell death. Indeed, there may be interactions between these positive and negative systems for regulating cell death. For example, Hattori *et al.* (1993) have suggested that TNFα stimulates the production of NGF in glial cells. Experiments designed to test how positive and negative regulators might interact to regulate the overall extent of programmed cell death will be useful in deciphering the mechanisms of programmed cell death in the developing nervous system.

It will be necessary to utilise neural cell cultures (Kalcheim and Neufield, 1990) to perform functional studies on the effects of TNFα, anti-TNFα, and antibodies to TNFRs on, for example, cell death as assessed by TUNEL labelling of cultures, and proliferation, as assessed by BrdU incorporation or PCNA staining (Sanders *et al.*, 1993). An *in vivo* method (Kashihara *et al.*, 1993) could also be used to apply TNFα, anti-TNFα, anti-TNFR1 and anti-TNFR2 onto the chorioallantoic membrane, through a window in the egg shell. After reincubation, the numbers of motoneurons in the ventral horn of the neural tube can be counted and assessed for TUNEL labelling in each condition.

As mentioned above, there is also evidence provided here that TNFα immunoreactivity is present in regions of the nervous system that clearly do not exhibit cell death; for example, the marginal zone of the neural tube, which contains axons rather than cell bodies. It is not clear from the present studies if TNFα immunoreactivity in the marginal zone is associated with glia or other non-neuronal cells. This is something which merits further attention, since TNFα has been shown to influence glial gene expression and function (Benveniste and Benos, 1995). There is also evidence that TNFα can induce Eck, a ligand for an Eph-related receptor with a role in the control of axon guidance (reviewed by Brambilla and Klein, 1995), thus implicating TNFα in this process. Clearly, TNFα may have roles other than in programmed cell death in the developing nervous system, such as those proposed in Chapter 3 in cell proliferation and differentiation.

Thus, in conclusion, future studies should be aimed at more detailed analyses of the particular cell types in the developing nervous system within which TNFα is expressed and

functional studies should be performed using *in vitro* systems to analyse TNFα-mediated effects on various cell processes in the nervous system; e.g. cell proliferation, differentiation, and migration.

8.9.2. TNFα in the limb buds

TNFα immunoreactivity is present in the limb buds and TUNEL labelling was revealed in the "necrotic" zones (Sanders and Wride, 1995) in the chick limb buds (see Chapters 4 and 5). Although a direct correlation between the presence of TUNEL labelled cells and the presence of TNFα immunoreactivity was not observed, the potential influence of TNFα-like factors on limb bud development deserves to be addressed further. This is particularly important in the light of the above speculations that thalidomide defects may be related to the effects of this drug on developmentally regulated TNFα expression in the limb buds and/or the mesonephros. In addition, TNFRs have been localised to regions of limb bud cell death (Wride and Sanders, unpublished). Regions of limb bud mesodermal tissue, destined to undergo programmed cell death, should be cultured (Ahrens *et al.*, 1977) and similar experiments to those described above, for nervous tissue, should be carried out. In addition, it will be interesting to see if TNFα influences any of the recognised effects of FGF and other factors on limb bud development (Tabin, 1995); for example on limb bud induction and chondrogenesis.

8.9.3. TNFα in myogenesis

TNFα has been found localised both to the myotome and developing cardiac muscle and TUNEL labelling is observed in these tissues (see Chapters 4 and 5). In addition, TNFR1 is also expressed at high levels in the myotome (Wride and Sanders, unpublished). Since programmed cell death of muscle occurs during normal development, this may be related to muscular dystrophy in the mammal and chick, since muscular dystrophy can be considered to be a consequence of dysregulated timing and extent of programmed cell death during muscle

development (Webb, 1974). For example, muscular dystrophy can be considered to be a normal process, but one which occurs too late to perform its normal developmental role or it could result from a failure of cell death to be switched off at the correct time in developing muscle. It will be important to investigate distributions of TNFα-like proteins and programmed cell death at various stages of muscle development and to determine if there is a functional link between TNFα expression and programmed cell death in developing muscle at particular stages. Culture of chick myotome cells (Sanders and Cheung, 1988) will be useful in elucidation of the roles for TNFα in early muscle development, especially since TNFα inhibits human myogenesis *in vitro* (Miller *et al.*, 1988) and has effects on the expression of CAMs and integrins (see Chapter 3), which may have roles in muscle differentiation (Rosen *et al.*, 1992).

8.9.4. The lens

So far, studies on potential effects of TNFα in the lens have focused on its role in lens fibre cell denucleation. However, in light of the hypothesis put forward in Chapter 3 and the usefulness of the developing lens as a model system for many processes occurring during embryonic development (see Chapter 6), it will be useful to evaluate the effects of TNFα on other aspects of chick lens development. For example, in Chapter 7 it was noted that TNFα exerts a dose-dependent effect on the extent of lentoid differentiation at early stages of lens epithelial cell differentiation, which may be related to its effects on lens epithelial cell proliferation. Furthermore, N-CAM is expressed in the lens, antibodies to N-CAM have effects on lens development (Watanabe *et al.*, 1989; see Chapter 6), and TNFα has been shown to be involved in upregulation of N-CAM expression (Vargas *et al.*, 1994). In addition, TNFα has been shown to inhibit gap junctional intercellular communication (Mensink *et al.*, 1995), which is very important in the lens (see Chapter 6). Thus, the developing lens will continue to provide an excellent model system for the study of many of the potential effects of TNFα during embryonic development.

8.10. Conclusions

This thesis has provided evidence that TNFα-like proteins are expressed during early stages of avian embryonic development in specific tissues that are undergoing important morphogenetic processes. The results suggest that proteins with TNFα like activities may have important roles in several processes occurring in these tissues, including regulation of rates of cell proliferation, remodelling of ECM, regulation of CAM and integrin expression, and in programmed cell death. The studies described here focused on potential roles for TNFα-like proteins in programmed cell death and the related process of lens fibre cell denucleation and studies utilising lens epithelial cells differentiating *in vitro* showed that TNFα specifically influences the latter process.

Thus, TNFα-like proteins have an important role in avian embryogenesis and future studies, some of which are suggested here to further investigate the role of TNFα during embryogenesis, are highly recommended. These studies will provide data of fundamental biological importance and may also lead to insights into, and treatments for, a number of diseases and congenital abnormalities, in which TNFα may be implicated.

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APPENDIX I: Data for Chapter 5

AI-1. Data for L929-8 bioassay.
Values for homogenate +/- anti-TNF antibodies

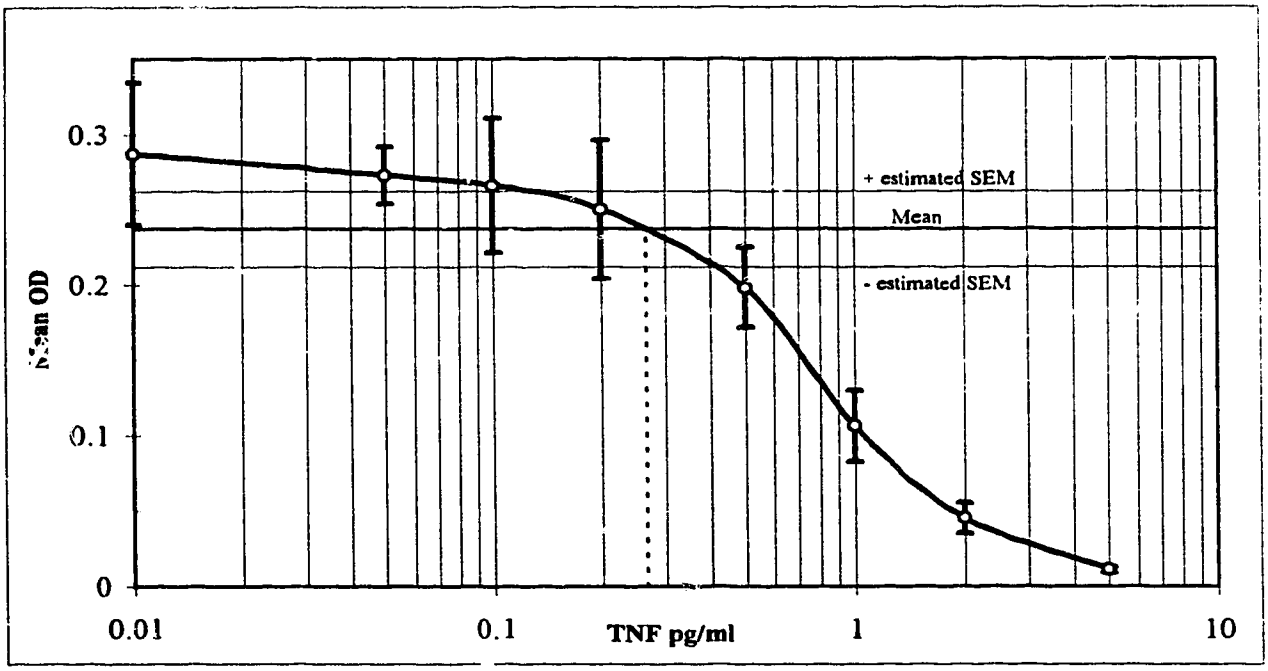
Condition	Homogenate alone	Ab 1	Ab 2
OD 1	0.227	0.434	0.347
OD 2	0.245	0.396	0.372
OD 3	0.248	0.415	0.494
OD 4	0.297	0.41	0.372
OD 5	0.284		
OD 6	0.123		

AI-2. Summary of T-test results for L929-8 bioassay.

Comparison	p
Homogenate & Ab1	0.00058
Homogenate & Ab2	0.00817

AI-3. Data for L929-8 bioassay.
Values for standard curve

Condition	Conc TNF ng/ml							
	0.01	0.05	0.1	0.2	0.5	1	2	5
OD 1	0.28	0.297	0.276	0.247	0.155	0.077	0.03	0.01
OD 2	0.381		0.342	0.324	0.182	0.099	0.043	0.014
OD 3	0.277	0.273	0.284	0.282	0.217	0.115	0.041	0.01
OD 4	0.265	0.284	0.244	0.234	0.204	0.146	0.061	0.011
OD 5	0.248	0.267	0.226	0.204	0.198	0.104	0.046	0.008
OD 6	0.272	0.246	0.225	0.208	0.231	0.093	0.048	0.012
Mean OD	0.287	0.273	0.266	0.25	0.198	0.106	0.045	0.011
Max	0.381	0.297	0.342	0.324	0.231	0.146	0.061	0.014
Min	0.248	0.246	0.225	0.204	0.155	0.077	0.03	0.008
n	6	5	6	6	6	6	6	6
St. Dev.	0.047	0.019	0.045	0.046	0.027	0.023	0.010	0.002



Standard curve of mean OD against concentration of TNF pg/ml for L929-8 bioassay

APPENDIX II: Data for Chapter 7

AII-1. Data for 2 day lens culture experiments. 1. 0-100 ng/ml TNF

TNF0 ng/ml			TNF 10 ng/ml			TNF 50 ng/ml			TNF 100 ng/ml		
area	# nuclei	nuc/area	area	# nuclei	nuc/area	area	# nuclei	nuc/area	area	# nuclei	nuc/area
57103	29	5.08	86316	35	4.05	58078	28	4.82	33573	32	9.53
66659	23	3.45	78379	39	4.98	59894	34	5.68	24025	19	7.91
21497	8	3.72	103790	54	5.20	69708	22	3.16	36543	21	5.75
93237	20	2.15	76952	36	4.68	41838	26	6.21	41560	26	6.26
63166	21	3.32	103810	35	3.37	43038	24	5.58	58737	17	2.89
70775	27	3.81	82268	38	4.62	58048	19	3.27	44113	13	2.95
62857	12	1.91	58551	23	3.93	57845	17	2.94	45620	40	8.77
98274	21	2.14	51520	13	2.52	66852	19	2.84	54999	30	5.45
98866	29	2.93	74482	20	2.69	45812	21	4.58	36318	27	7.43
55169	41	7.43	44618	22	4.93	64590	22	3.41	38419	26	6.77
68834	41	5.96	39806	26	6.53	62416	20	3.20	53621	41	7.65
103490	22	2.13	49762	26	5.22	44333	30	6.77	65934	42	6.37
70936	27	3.81	67535	19	2.81	45742	19	4.15	64263	28	4.36
77408	17	2.20	75024	25	3.33	34944	24	6.87	42417	37	8.72
87208	25	2.87	66754	23	3.45	65067	30	4.61	55360	42	7.59
93116	21	2.26	45047	18	4.00	50040	29	5.80	46625	48	10.29
93555	45	4.81	61612	17	2.76	67512	21	3.11	39595	32	8.08
86179	25	2.90	45988	22	4.78	37609	35	9.31	50226	34	6.77
71288	17	2.38	58421	19	3.25	63326	24	3.79	38127	21	5.51
105390	47	4.46	57899	23	3.97	63850	24	3.76	48931	27	5.52
57467	27	4.70				58788	21	3.57	78093	60	7.68
125310	17	1.36				47328	29	6.13	45558	23	5.05
102940	34	3.30				65829	30	4.56			
90267	18	1.99				40930	31	7.57			
101570	21	2.07				42337	26	6.14			
89688	36	4.01				41939	20	4.77			
75027	38	5.06				40127	20	4.98			
142240	19	1.34				30686	18	5.87			
134360	28	2.08				70739	22	3.11			
50028	16	3.20				66794	28	4.19			
96218	24	2.49				68165	21	3.08			
						45836	24	5.24			
						61093	23	3.76			
						52669	21	3.99			
						72882	22	3.02			

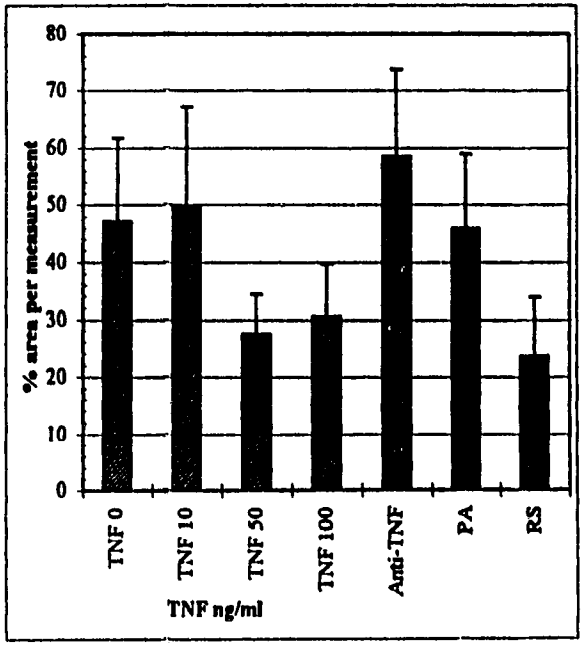
AII-2. Data for 2 day lens culture experiments. 2. Anti TNF

Anti-TNF 1:250			*Pre Abs Cont			RS Cont 1:250		
area	# nuclei	nuc/area	area	# nuclei	nuc/area	area	# nuclei	nuc/area
95571	1	0.10	77603	10	1.29	35188	26	7.39
70807	7	0.99	52719	11	2.09	39039	14	3.59
124510	3	0.24	74908	14	1.87	59246	16	2.70
74819	4	0.53	44372	6	1.35	49348	9	1.82
66553	3	0.45	75657	15	1.98	11902	8	6.72
67956	8	1.18	61343	9	1.47	65710	8	1.22
12399	1	0.81	86453	11	1.27	32539	11	3.38
77453	3	0.39	83922	14	1.67	46451	15	3.23
94106	4	0.43	32995	0	0.00	55098	21	3.81
140280	3	0.21	78042	10	1.28	10607	8	7.54
173360	9	0.52				53351	15	2.81
124430	2	0.16						
91604	3	0.33						
116050	5	0.43						
58772	4	0.68						
77538	5	0.64						
94602	12	1.27						
73238	5	0.68						
80839	4	0.49						
96364	10	1.04						

*1:250 anti-TNF/50 ng/ml TNF

AII-3. Data for 2 day lens culture experiments. 3. % area of lentoids

TNF 0	TNF 10	TNF 50	TNF 100	Anti-TN	PA	RS
31.5	61.6	32.7	14.7	52.8	60.9	19.4
36.8	43.3	25.3	20.4	39.1	46.5	12.9
11.9	57.3	19.2	14.1	78.4	50.8	21.6
51.5	48.3	22.4	22.1	41.3	32.4	32.7
34.9	57.1	27.6	31.2	47.7	41.8	27.3
39.1	57.8	37.3	40.7	72.2	58.4	9.6
34.7	57.4	20.8	37.0	68.5	49.9	36.3
65.1	29.7	35.0	36.6	57.6	57.5	18.0
54.6	47.4	28.6	29.9	55.6	18.2	25.7
30.5	35.2	32.5	30.4	83.7	43.1	30.4
38.0	37.6	26.1	20.1	95.8		8.7
57.2	87.9	36.4	21.2	68.7		40.3
39.2	83.3	22.6	29.6	57.7		
42.8	56.8	23.4	42.2	64.1		
48.2	36.9	22.2	38.1	47.5		
51.4	24.9	16.9	27.6	50.6		
51.7	34.0		37.8	52.3		
47.6	25.4		26.9	40.5		
39.4	44.7		31.4	44.6		
58.2	69.2		32.0	53.2		
31.7			27.1			
69.2			39.1			
56.9			52.3			
49.9			32.0			
56.1						
49.5						
41.4						
78.6						
74.2						
27.6						
62.6						



T-Test Results: % lentoid area	
Comparison	p
TNF 0 & TNF 10	0.5810
TNF 0 & TNF 50	0
TNF 0 & TNF 100**	0
TNF 10 & TNF 50**	0
TNF 10 & TNF 100**	0.0001
TNF 50 & TNF 100	0.1023
Anti-TNF & PA*	0.0282
Anti-TNF & RS**	0
PA & RS**	0.0004
Anti-TNF & TNF 0*	0.0115

Notes: * $p < 0.05$; ** $p < 0.01$

AII-4. Data for 2 day lens culture experiments. 4. TNFR1 and TNFR2.

TNF 0 ng/ml			TNFR1			TNFR2		
area	# nuclei	nuc/area	area	# nuclei	nuc/area	area	# nuclei	nuc/area
122980	41	3.33	149670	45	3.01	182690	101	5.53
135630	82	6.05	172760	127	7.35	119870	45	3.75
153200	139	9.07	172570	109	6.32	166940	129	7.73
184640	35	1.90	148200	75	5.06	150550	123	8.17
105380	20	1.90	127200	73	5.74	163270	200	12.25
118440	49	4.14	133890	92	6.87	162930	107	6.57
150210	55	3.66	119850	79	6.59	123330	68	5.51
137770	88	6.39	163100	105	6.44	140700	131	9.31
152150	47	3.09	134200	38	2.83	115550	59	5.11
175100	25	1.43	105230	102	9.69	152740	133	8.7'
169880	50	2.94	143310	96	6.70	105180	97	9.22
160190	48	3.00	150030	44	2.93	153050	145	9.47
143410	24	1.67	109440	56	5.12	160730	109	6.78
177350	65	3.67	141680	83	5.86	179990	114	6.33
152230	59	3.88	159650	148	8.72	168080	167	9.94
154600	25	1.62	153200	134	8.75	171990	94	5.47
143770	88	6.12	163140	85	5.21	158150	104	6.58
118840	41	3.45	144370	104	7.20	124160	85	6.85
87727	46	5.24	150010	154	10.27	104540	71	6.79

AII-5. Data for 4 day lens culture experiments.

TNF 0 ng/ml			TNF 10 ng/ml			TNF 50 ng/ml			TNF 100 ng/ml			Anti-TNF		
area	# nuclei	nuc/area	area	# nuclei	nuc/area	area	# nuclei	nuc/area	area	# nucell	nuc/area	area	# nuclei	nuc/area
74478	29	3.89	37976	9	2.37	66539	39	5 86	78028	22	2 82	68459	2	0.29
65091	22	3 38	52952	26	4.91	36974	16	4.33	44274	33	7.45	56705	0	0.00
71688	12	1.67	67730	11	1.62	37871	14	3.70	71340	22	3.08	91689	3	0.33
69445	22	3.17	26562	9	3.39	39504	20	5.06	46737	23	4.92	65448	2	0.31
73452	24	3.27	66739	28	4.20	56220	34	6.05	70199	27	3.85	113660	0	0.00
69344	18	2.60	73133	24	3.28	39995	40	10.00	52719	39	7.40	53977	0	0.00
63722	20	3.14	53328	12	2 25	44825	26	5.80	42642	23	5.39	59678	1	0.17
92016	25	2.72	56918	8	1.41	54174	31	5.72	48453	21	4.33	65076	0	0.00
77803	33	4.24	45924	16	3.48	58139	24	4.13	13886	14	10.08	78669	2	0.25
88329	36	4 08	74176	12	1 62	48757	20	4.10	32379	16	4.94	74668	0	0.00
77280	30	3.88	28036	12	4.28	39990	14	3.50	44039	29	6.59	25031	0	0.00
50156	38	7.58	77364	13	1 68	33395	21	6 29	57087	39	6.83			
41298	8	1.94	57410	22	3 83	62043	32	5.16	67713	26	3.84			
43556	9	2 07	62960	31	4.92	48088	42	8.73	52766	40	7.58			
64100	29	4 52	52326	27	5 16	46537	26	5.59	45115	19	4.21			
72400	14	1.93	60531	19	3.14	38040	31	8 15	33971	32	9.42			
40167	24	5.98	68727	23	3.35	53607	29	5.41	51268	18	3.51			
78054	15	1.92	54491	24	4.40	57258	18	3.14	77560	32	4.13			
51510	12	2 33	64464	18	2.79	63683	20	3.14	44863	17	3.79			
44626	26	5.83	47558	19	4.00	58370	24	4.11	44604	21	4.71			
66208	27	4 08	53768	31	5.77	55694	47	8.44	75938	33	4.35			
62831	13	2.07	49661	22	4.43	61668	44	7.13	67670	56	8.25			
55839	16	2.87	50844	45	8.85	88666	20	2.26	69829	31	4.44			
75504	34	4.50	72203	41	5.68	87809	26	2.96	62245	32	5.14			
			53762	23	4.28	54506	43	6 67	58222	29	4.98			
			41711	14	3.36	78261	36	4.60	70490	26	3.69			
			45355	7	1.54	30503	42	14.75	76036	27	3.55			
			51729	24	4.64	44386	56	12.62	69161	64	9.25			
			69192	39	5 64	54999	47	8.55	65153	26	3.99			
			64983	34	5.23	62390	34	5.45	65525	40	6.10			
			26033	7	2.69	53880	52	9.65	53794	23	4.28			
			61020	29	4.75	71124	69	9.70	45211	18	3.98			
			54790	31	5.66	41589	39	9.38	70869	27	3 81			
			60629	28	4.62	52000	49	9.42	56428	20	3.54			
			48611	15	3.09	49195	32	6.50	59338	23	3.88			
			48117	27	5.61									
			53135	20	3.76									
			70240	30	4.27									
			60478	34	5.62									
			66664	32	4.80									
			78155	32	4.09									
			99556	43	4.32									
			30782	7	2.27									
			64187	21	3.27									

AII-6. Data for 6 day lens culture experiments

TNF 0 ng/ml			TNF 10 ng/ml			TNF 50 ng/ml			TNF 100 ng/ml		
area	# nuclei	nuc/area	area	# nuclei	nuc/area	area	# nuclei	nuc/area	area	# nuclei	nuc/area
49678	19	3.82	64004	25	3.91	60311	25	4.15	60311	8	1.33
31952	15	4.69	76413	21	2.75	61072	17	2.78	61072	7	1.15
33238	3	0.90	40414	39	9.65	47445	19	4.00	47445	9	1.90
52754	8	1.52	67352	50	7.42	54426	49	9.00	54426	12	2.20
26947	19	7.05	54370	21	3.86	51801	23	4.44	51801	13	2.51
28666	7	2.44	66190	34	5.14	66414	32	4.82	66414	22	3.31
51320	15	2.92	67562	12	1.78	48932	45	9.20	48932	8	1.63
71570	25	3.49	63137	25	3.96	59348	52	8.76	59348	28	4.72
61747	22	3.56	50854	20	3.93	29470	28	9.50	29470	7	2.38
42815	13	3.04	57947	41	7.08	61446	63	10.25	61446	10	1.63
10242	6	5.86	48298	25	5.18	47060	21	4.46	47060	0	0.00
72401	33	4.56	49235	27	5.48	44233	5	1.13	44233	17	3.84
77505	21	2.71	57047	48	8.41	63941	53	8.29	63941	27	4.22
81174	32	3.94	36531	15	4.11	57698	56	9.71	57698	48	8.32
64144	93	14.50	41655	19	4.56	65507	69	10.53	65507	38	5.80
50430	33	6.54	29842	19	6.37	48764	48	9.84	48764	13	2.67
43563	37	8.49	48053	21	4.37	71377	66	9.25	71377	24	3.36
92179	58	6.29	61456	16	2.60	90215	86	9.53	90215	27	2.99
52315	22	4.21	57776	26	4.50	56500	57	10.09	56500	23	4.07
62342	29	4.65	27380	14	5.11	70977	45	6.34	70977	32	4.51
39449	13	3.30	55479	38	6.85	54779	55	10.04	54779	21	3.83
68331	23	3.37	55062	28	5.09						
70284	21	2.99	51200	21	4.10						
74242	49	6.60	75092	60	7.99						
70027	42	6.00	56864	22	3.87						
34694	26	7.49	63663	23	3.61						
88328	58	6.57	52707	19	3.60						
66734	21	3.15	55236	37	6.70						
47638	17	3.57									
65556	28	4.27									
73751	38	5.15									
62217	60	9.64									

AII-7. Summary of T-test results: nuclei/area

2 day lens culture		Anti TNFR1 & TNFR2	
Comparison	**P**	**Comparison**	**p**
TNF 0 & TNF 10*	0.0279	TNF0 & R1**	0.0006
TNF 0 & TNF 50**	0.0002	TNF0 & R2**	0.0000
TNF 0 & TNF 100**	0.0000	R1 and R2	0.1045
Anti-TNF & PA**	0.0012		
Anti-TNF & RS**	0.0004		
Anti-TNF & TNF 100**	0.0000		
TNF 10 & TNF 50	0.0795		
TNF 10 & TNF 100**	0.0000		
TNF 50 & TNF 100**	0.0002		
PA & RS**	0.0028		
Anti-TNF & TNF 0**	0.0000		
4 day lens culture		**6 day lens culture**	
Comparison	**p**	**Comparison**	**p**
TNF 0 & TNF 10	0.2083	TNF 0 and TNF 10	0.7920
TNF 0 & TNF 50**	0.0000	TNF 0 and TNF 50**	0.0029
TNF 0 & TNF 100**	0.0003	TNF 0 and TNF 100**	0.0090
TNF 0 & Anti-TNF**	0.0000		
TNF 10 & TNF 50**	0.0000		
TNF 10 & TNF 100**	0.0025		
TNF 10 & Anti-TNF**	0.0000		
TNF 50 & TNF 100*	0.0347		
TNF 100 & Anti-TNF**	0.0000		

Notes: * $p < 0.05$; ** $p < 0.01$

AII-8. Summary of T-test results: area

2 day lens culture		Anti TNFR1 & TNFR2	
Comparison	**p**	**Comparison**	**p**
TNF 0 & TNF 10**	0.0061	TNF0 & R1	0.7886
TNF 0 & TNF 50**	0.0000	TNF0 & R2	0.5908
TNF0 & TNF 100**	0.0000	R1 & R2	0.6846
Anti-TNF & PA*	0.0187		
Anti-TNF & RS**	0.0000		
Anti-TNF & TNF 100**	0.0000		
TNF 10 & TNF 50**	0.0154		
TNF 10 & TNF 100**	0.0005		
TNF 50 & TNF 100*	0.0373		
PA & RS**	0.0084		
Anti-TNF & TNF 0	0.4780		
4 day lens culture		**6 day lens cultures**	
Comparison	**p**	**Comparison**	**p**
TNF 0 & TNF 10*	0.0255	TNF 0 and TNF 10	0.6022
TNF 0 & TNF 50**	0.0033	TNF 0 and TNF 50	0.8401
TNF 0 & TNF 100*	0.0291	TNF 0 and TNF 100	0.8254
TNF 0 & Anti-TNF	0.6821	TNF 10 & TNF 50	0.39387847
TNF 10 & TNF 50	0.3270	TNF 10 & TNF 100	0.40354546
TNF 10 & TNF 100	0.9537	TNF 50 & TNF 100	0.99380477
TNF 10 & Anti-TNF	0.1290		
TNF 50 & TNF 100	0.3866		
TNF 100 & Anti-TNF	0.2933		

Notes: * $p < 0.05$; ** $p < 0.01$

AII-9. Summary of T-test results: nuclei

2 day lens culture		Anti TNFR1 & TNFR2	
Comparison	**p**	**Comparison**	**p**
TNF 0 & TNF 10	0.7299	TNF0 & R1**	0.0006
TNF 0 & TNF 50	0.4114	TNF0 & R2**	0.0000
TNF 0 & TNF 100	0.0677	R1 and R2	0.1045
Anti-TNF & PA**	0.0050		
Anti-TNF & RS**	0.0004		
Anti-TNF & TNF100	0.0000		
TNF 10 & TNF 50	0.2908		
TNF 10 & TNF 100	0.1709		
TNF50 & TNF 100**	0.0092		
PA & RS**	0.0000		
Anti-TNF & TNF 0**	0.0000		
4 day lens culture		**6 day lens cultures**	
Comparison	**p**	**Comparison**	**p**
TNF 0 & TNF 10	0.8753	TNF 0 and TNF 10	0.8137
TNF 0 & TNF 50**	0.0003	TNF 0 and TNF 50**	0.0097
TNF 0 & TNF 100*	0.0232	TNF 0 and TNF 100*	0.0289
TNF 0 & Anti-TNF**	0.0000	TNF 10 & TNF 50**	0.0030
TNF 10 & TNF 50**	0.0003	TNF 10 & TNF 100*	0.0159
TNF 10 & TNF 100*	0.0232	TNF 50 & TNF 100**	0.0000
TNF 10 & Anti-TNF	0.0000		
TNF 50 & TNF 100**	0.0885		
TNF 100 & Anti-TNF**	0.0000		

Notes: * $p < 0.05$; ** $p < 0.01$

APPENDIX III: List of publications at time of submission of thesis

MANUSCRIPTS IN PREPARATION OR SUBMITTED.

Wride M A, Sanders E J. (1996). TNFα and its receptors in the chick embryo lens: effects on degeneration of lens fibre cell nuclei. **article**

Wride M A. (1996). Cellular and molecular features of lens development: a review of recent advances. **review**

Sanders E J, Prasad S, Hu N, Wride M A. (1996). Cell death in the gastrulating chick embryo: potential roles for tumour necrosis factor-alpha (TNF-alpha). (submitted) **article**

ARTICLES/REVIEWS PUBLISHED OR IN PRESS.

Sanders E J, Wride MA. (1996). Roles for growth and differentiation factors in avian development. *Poultry Sci.*, (in press). **review**

Sanders E J, Wride M A. (1995). Ultrastructural identification of apoptotic nuclei using the TUNEL technique. *J. Histochem,*. (in press). **article**

Sanders E J, Wride M A. (1995). Programmed cell death in development. *Int. Rev. Cytol.*, 163: 105-173. **review**

Wride M A, Sanders E J. (1995). Potential roles for tumour necrosis factorα during embryonic development. *Anat. Embryol.* 191: 1-10. **review**

Wride M A, Lapchak P H, Sanders E J. (1994). Distribution of TNFα-like proteins correlates with some regions of programmed cell death in the chick embryo. *Int. J. Dev. Biol.*, 38: 673-682. **article**

Sanders E J, Hu N, Wride MA. (1994). Expression of TGFß1 during early chick embryo development. *Anat. Rec.*, 238: 397-406. **article**

Wride M A, Sanders EJ. (1993). Expression of tumor necrosis factor-alpha (TNFα) cross-reactive proteins during early chick embryo development. *Dev. Dynamics*, 198: 225-239. **article**

Grimble R F, Jackson A A, Persaud C, Wride M A, Delers F, Engler R. (1992). Cysteine and glycine supplementation modulate the metabolic response to tumor necrosis factor α in rats fed a low protein diet. *J. Nutrition*, 122: 2066-2973. **article**

ABSTRACTS.

Sanders E J and Wride M A (1996). Potential roles for TNFα in early avian embryonic development. 6th International TNF congress. Rhodes, Greece. **poster**

Johnson C D M, Wride M A, Hull K L, Harvey S (1996). Immunohistochemical localization of growth hormone and growth hormone receptor in the early chick embryo. Proceedings of the VI International Symposium on Avian Endocrinology, Lake Louise, Canada. **poster**

Wride M A and Sanders E J (1996). Potential roles for tumor necrosis factor-alpha in the development of the chick embryo lens. Proceedings of the VI International Symposium on Avian Endocrinology, Lake Louise, Canada. **invited speaker**

Wride M A, Sanders E J. (1995). TNF-α and its receptors in the developing avian lens. *Mol. Biol. Cell,* 323a. **poster**

Wride M.A, Sanders E J. (1994). Immunocytochemical localisation of tumour necrosis factor-α (TNF-α) and TNF-receptors (TNFR1 and TNFR2) in chick eye lenses and in lens epithelial explants *in vitro*. Brit. Soc. Dev. Biol. Autumn Meeting, Durham, U.K. **poster**

Wride M A, Sanders E J (1993). Programmed cell death in chick embryo development: a study using TdT-mediated dUTP-biotin nick end-labeling (TUNEL) and correlation with expression of tumor necrosis factor-alpha (TNFα) cross-reactive proteins. *Mol. Biol. Cell,* 4s: 143a. **poster**

Wride M A, Sanders E J. (1992). Immunocytochemical localization of tumor necrosis factor-alpha (TNF-α) during early chick embryo development. *Mol. Biol. Cell,* 3s: 109a.. **poster**

Grimble R F, Jackson A A, Persaud C, Wride M A, Delers F, Engler R. (1991). Modulatory effects of glycine and cysteine on the response of glutathione and acute phase proteins to tumour necrosis factor α in rats. *Proc. Nutr. Soc.,* 50: 169a.