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UNIVERSITY OF ALBERTA

ROLE OF BCL-2 IN PLACENTAL TROPHOBLAST APOPTOSIS

By

SAMUEL KIN SANG HO

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE.**

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **THE ROLE OF BCL-2 IN PLACENTAL TROPHOBLAST APOPTOSIS** by **SAMUEL KIN SANG HO** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**.

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ABSTRACT

Bcl-2 is a protein that protects cells from apoptosis induced by a variety of stimuli, including TNF-α. It is expressed in many different tissues, with expression levels being the highest in the fetal placental trophoblasts. It has been shown previously that TNF-α induces trophoblast apoptosis in an EGF-suppressible manner. Based on this, it was hypothesized that TNF-α-induced apoptosis is associated with down-regulation of Bcl-2 while EGF protects against apoptosis by up-regulating Bcl-2.

Upon testing, this hypothesis was found to be incorrect, since Bcl-2 protein levels did not change in response to EGF or TNF-α. Other agents which influenced trophoblast survival such as cycloheximide, GM-CSF, and the glucocorticoid dexamethasone also did not affect Bcl-2 protein expression. The expression levels did not vary with the developmental or differentiation state of the trophoblasts. These data suggest that endogenous expression of Bcl-2 is constitutive and stable.

However, trophoblasts which expressed lower levels of Bcl-2 were found to be predisposed to TNF-α-induced apoptosis compared to cells with higher levels of the protein. This indicates functional heterogeneity amongst trophoblasts. Transfection studies aimed at modulating Bcl-2 protein levels were unsuccessful.

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ABBREVIATIONS

Apaf	Apoptosis activating factor
BH	Bcl-2 homology domain
BSA	Bovine serum albumin
BSSS	Balanced salt solution with 0.1% (w/v) saponin
CHX	Cycloheximide
CMV	Cytomegalovirus
CrmA	Cytokine response modifier A
CT	Cytotrophoblast
DDW	Deionized-distilled water
Dex	Dexamethasone
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
HLA	Human leukocyte antigens
HRP	Horse radish peroxidase enzyme
ICE	Interleukin-1β converting enzyme
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IUGR	Intrauterine growth retardation
kb	Kilobase
kDa	KiloDalton

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NRE	Negative regulatory element
ORF	Open reading frame
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBSA	PBS containing 1% (w/v) bovine serum albumin and 0.02% (w/v) NaN_3
PCD	Programmed cell death
rHu-	Human recombinant protein
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
ST	Syncytiotrophoblast
TdT	Terminal deoxynucleotidyl transferase
TNF-α	Tumor necrosis factor alpha
TNF-R1	Tumor necrosis factor receptor p55
TUNEL	TdT-mediated dUTP-biotin nick end labeling
WB	Wash buffer for tissue sections
WBB	Western blot buffer

CHAPTER 1

INTRODUCTION

1.1 PHYSIOLOGICAL CELL DEATH

An essential component of tissue homeostasis involves a balance between the production of new cells and the removal of old cells. Excess cell proliferation often contributes to the disease state of cancer, while excess cell death results in degenerative diseases such as Alzheimer's disease. Immense interest in research to study cell proliferation has occurred in the last three decades, the outcome of which has greatly advanced our understanding of the molecular mechanisms of cell growth. Conversely, physiological cell death has been neglected as an important element of cellular biology until recently. Only in the last ten years has there been a surge of scientific interest in understanding the role of cell death in normal physiology and in disease states.

Function and features of programmed cell death and apoptosis

Some of the many physiological functions of cell death include the elimination of cells which are either non-functional, produced in excess, improperly developed, or are approaching the end of their life-spans (Ellis *et al.*, 1991). Cell death is also utilized in the production of dead cells for special functions, for instance cornification and lens formation (Fesus *et al.*, 1991). During many viral infections, physiological cell death is induced, either by viral components, by elements of the immune system such as cytokines and immune cells, or initiated by the infected cells themselves as a means of limiting tissue damage. The way by which this cell death occurs is a special process called programmed cell death (PCD). The term refers to the fact that the dying cell is actively involved in its own demise (Wyllie *et al.*, 1981; Martin *et al.*, 1988; Kizaki *et al.*, 1989; McConkey *et al.*,1989; Odaka *et al.*, 1990; Rawson *et al.*, 1990; Schwartz *et al.*, 1990; Ju, 1991; Vaux, 1993). It was also found that

macromolecular-synthesis inhibitors are unable to protect cells against PCD, indicating the presence of a pre-existing machinery within cells for the execution of cell death (Edwards *et al.*, 1991; Sellins and Cohen, 1991; Dutz *et al.*, 1992; Lin and Chou, 1992).

The term apoptosis (Kerr *et al.*,1972) has been coined to describe the collective cellular morphology during PCD. This form of cell death usually affects scattered individual cells instead of whole tissues, as in necrosis, which almost always results in tissue damage and ensuing inflammatory response. Apoptosis often occurs rapidly, with the dying cell disappearing within 4 hours (Fesus *et al.*, 1991; Schwartzman and Cidlowski, 1993). Characteristically, the apoptotic cell cytoplasm shrinks and the chromatin condenses and compacts against the nuclear membrane to manifest as several dark patches under the microscope (Wyllie *et al.*, 1980; Kerr and Harmon, 1991). Organelles also compact, although morphologically mitochondria and lysosomes stay normal. The nucleolus disintegrates and the nucleus also decreases in size, with the eventual breakdown of the nuclear envelope and fragmentation of the nucleus. Accompanied by cytoskeletal rearrangement and plasma membrane blebbing, the cell develops cytoplasmic processes containing the nuclear fragments (Kerr and Harmon, 1994). When they finally separate from the remainder of the cell, these form apoptotic bodies which are then phagocytosed by neighboring cells or macrophages. Sometimes the whole cell may condense into one single apoptotic body. Because the plasma membrane remains structurally intact throughout this process, there is no leakage of cytoplasmic material which could cause damage to neighboring cells and elicit an inflammatory response. Another mechanism by which neighboring cells are protected is the apoptotic cell's early loss of cell junctions. The outcome of this is either the loss of cell adhesion or the inability of PCD-signaling molecules to diffuse into adjacent healthy cells. This is in sharp contrast with

another form of cell death, necrosis. Neighboring cells are affected by necrotic cells (Searle *et al.*, 1982), due to a loss of membrane integrity and leakage of cytoplasmic contents that include activated lysosomal enzymes. The organelles are damaged and the cytoplasm typically swells and becomes fragmented. The chromatin is also fragmented, accompanied by nuclear swelling. Consequently the summation of these extensive cellular injuries is an infiltration of inflammatory cells and further tissue damage. Table 1.1 summarizes the differences between apoptosis and necrosis.

One hallmark biochemical change within apoptotic cells is the internucleosomal cleavage of DNA into 200 bp fragments (Wyllie *et al.*, 1981; Arends *et al.*, 1990) by a endoplasmic reticulum (ER) residing, Ca^{2+}/Mg^{2+}-dependent endonuclease (Arends *et al.*, 1990; Lam *et al.*, 1994) that is functionally and antigenically identical to DNase I (Peitsch, *et al.*, 1993). Nuclear lamin is phosphorylated and solubilized, accounting for nuclear envelope breakdown and access of DNase I to DNA. Some cell types undergoing apoptosis do not exhibit internucleosomal DNA degradation, implying DNA cleavage does not initiate apoptosis but is rather a means of DNA disposal (Cohen *et al.*, 1992; Ucker *et al.*, 1992; Oberhammer *et al.*, 1993; Peitsch *et al.*, 1994). Elevated intracellular Ca^{2+} concentration activates latent enzymes, such as transglutaminase (Wyllie, 1993), which may alter cytoadhesive properties of apoptotic cells (Fesus *et al.*, 1991). Immature glycans, normally buried in mature cell surface glycoproteins, are exposed for recognition by phagocyte receptors, of which vitronectin- and thrombospondin-receptors are two examples (Fesus *et al.*, 1991).

A special class of proteases in the cytoplasm is believed to be the executioner of apoptosis. These are the interleukin-1β converting enzyme (ICE) family of cysteine proteases or caspases (Alnemri *et al.*, 1996). ICE was originally identified in monocytes for its ability to process the 31 kDa inactive precursor of

IL-1β into its 17 kDa active form (Black *et al.*, 1989; Kostura *et al.*, 1989). Overexpression of either ICE or Ced-3, a nematode homologue from *Caenorhabditis elegans*, in Rat-1 fibroblasts induces apoptosis (Miura *et al.*, 1993). Other homologues, such as Nedd-2 (Kumar *et al.*, 1994), Ich-1 (Wang *et al.*, 1994), CPP32 (Fernandes-Alnemri *et al.*, 1994; Tewari *et al.*, 1995; Nicholson *et al.*, 1995), TX (Faucheu *et al.*, 1995), and Mch-2 (Fernandes-Alnemri *et al.*, 1995), also cause apoptosis in other cell lines. These are enzymes sharing the conserved pentapeptide QACRG, with the central cysteine being the catalytic residue. They have the unique substrate specificity of aspartate in the P1 position (Thornberry *et al.*, 1992). These proteases also undergo proteolytic cleavage to become activated. Interestingly, granzyme A and granzyme B, which are secreted apoptosis-inducing serine proteases of activated cytotoxic lymphocytes and NK cells, also share this substrate preference. However, only granzyme A can process IL-1β (Irmler *et al.*, 1995). Although it is not clear what biochemical roles these caspases play in apoptosis, some important substrates are nonetheless identified. A 70 kDa protein in the U1 small ribonucleoprotein particle that is essential for mRNA splicing is cleaved by an ICE-like protease (Casciola-Rosen *et al.*, 1994). Poly(ADP-ribose) polymerase (PARP), which is involved in DNA repair and supervision of genome structure and integrity in stressed cells (Nicholson *et al.*, 1995), is cleaved by CPP32 (Lazebnik *et al.* 1994; Fernandes-Alnemri *et al.*, 1994; Tewari *et al.*, 1995; Nicholson *et al.*, 1995) and Mch-2 (Fernandes-Alnemri *et al.*, 1995) during apoptosis. This cleavage is most likely to be an outcome instead of the initiator of apoptosis, since PARP knockout mice are developmentally normal (Wang *et al.*, 1995). Nicholson *et al.* (1995) also found that CPP32 alone, in contrast to cytosol extract containing CPP32, is unable to induce apoptotic features in isolated healthy nuclei, indicating CPP32 is essential but not sufficient for apoptosis, or a critical substrate is present only in the cytoplasm. In accord with

this, ICE is also not required for normal development in mice, suggesting redundancy of caspases in the control of apoptosis (Kuida *et al.*, 1995).

TNF-α as trigger of apoptosis

Of all the triggers of apoptosis, tumor necrosis factor-α (TNF-α) is one of the most intriguing. This is due to the well-documented fact that TNF-α has different effects on different cell types. Effects could be stimulation or inhibition of cell proliferation, cell death, activation or inhibition of cellular functions depending on the concentration, exposure time, and the presence of other agents that either antagonize or synergize with TNF-α (Camussi *et al.*, 1990; Heller and Krönke, 1994). This complexity is compounded by the observation that TNF receptors (p55 and p75) are expressed in most, if not all, cell types (Lewis *et al.*, 1991). Furthermore, caspases appear to be downstream effectors of TNF-α-mediated cell death. For instance, Mashima *et al.* (1995) found that TNF-α induced apoptosis in U937 human myeloid leukemia cells is blocked by a protease inhibitor that preferentially inhibits ICE and ICE-like proteases. Apoptosis mediated by overexpression of ICE in Rat-1 fibroblasts can be suppressed by cytokine response modifier A (CrmA; Miura *et al.*, 1993), a cowpox viral protein that represses caspases (Ray *et al.*, 1992; Komiyama *et al.*, 1994). Lastly, CrmA also suppresses TNF-α-mediated apoptosis (Tewari and Dixit, 1995; Miura *et al.*, 1995; Enari *et al.*, 1995). Recent biochemical studies have shed even more light on the connection between TNF-α and caspases. Homologous stretches of about 75 amino acids in the cytoplasmic tails of tumor necrosis factor receptor p55 (TNF-R1) and Fas called 'death domains' function as docks for protein-protein interaction with downstream signaling molecules (Tartaglia, *et al.*, 1993; Itoh and Nagata, 1993). In the case of TNF-R1, its death domain recruits FADD (for Fas-associated through death domain; Chinnaiyan *et al.*, 1996 a), which in turn recruits

FLICE (for FADD-like-ICE), an ICE-like protease capable of initiating downstream cascade of caspases (Boldin *et al.*, 1996; Muzio *et al.*, 1996). One of these is a close relative of CPP32, ICE-LAP3 (for ICE-like apoptotic protease 3), which is activated during TNF-α-induced apoptosis and can be inhibited by CrmA (Duan *et al.*, 1996). Although CPP32 itself has not been definitively demonstrated to be involved in TNF-α-induced apoptosis, there is conclusive evidence that it is an effector of Fas-mediated cell death and cleaves PARP upon activation (Schlegel *et al.*, 1996). Since both TNF-R1 and Fas enlist FADD and FLICE during apoptosis, there is a strong possibility that TNF-α also employs CPP32.

1.2 PROTECTION AGAINST PCD BY BCL-2

Cells do not passively respond to cell death signals and initiate apoptosis. More often than not the cell receiving cell death signals such as TNF-α have a part in the final decision of commitment to apoptosis. The mechanism by which this process occurs has slowly surfaced since the discovery of the gene that encodes the protein Bcl-2.

Discovery of the *bcl-2* gene

The *bcl-2* (for B-cell lymphoma/leukemia 2) gene was identified in 1985 at the breakpoint of chromosomal translocation t(14;18)(q24;q21) (Tsujimoto *et al.*, 1985; Bakhshi *et al.*, 1985; Cleary and Sklar, 1985). It is the most common genetic alteration in human myeloid tumors and occurs in 85% of follicular lymphomas, 20-30% of diffuse large cell lymphomas, and 50% of diffuse undifferentiated non-Burkitt lymphomas (Fukahara *et al.*, 1979; Lipford *et al.*, 1987; Yunis *et al.*, 1987). The translocation moves *bcl-2* from chromosome 18 into juxtaposition with the immunoglobulin (Ig) heavy chain gene locus on chromosome 14, and is likely

the outcome of erroneous DNA recombination during Ig VDJ gene rearrangement during B-cell differentiation (Tsujimoto *et al.*, 1988). This movement confers *bcl-2* transcriptional activity through the influence of powerful enhancers in the Ig locus with a corresponding elevation in protein level (Chen-Levy *et al.*, 1989).

Gene structure and protein localization of *bcl-2*

The *bcl-2* locus is 230 kb in length and consists of 3 exons and a 225 kb intron separating exons 2 and 3 which carry the open reading frame (ORF; Silverman *et al.*, 1990). A variably spliced alternative exon 1, the significance of which is unknown, is 5 kb upstream in the 5' untranslated region (Hua *et al.*, 1989). There are 2 promoters, P1 and P2, that regulate *bcl-2* expression, with P1 being predominantly used and P2, located just before the start of the ORF within exon 2, apparently infrequently used (Negrini *et al.*, 1987; Seto *et al.*, 1988). Interestingly, p53, a tumor suppressor and apoptosis inducer (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992), negatively regulates *bcl-2* transcription via a 1.3 kb negative regulatory element (NRE) between P1 and P2 (Young and Korsmeyer, 1993; Miyashita *et al.*, 1994).

Transcripts of *bcl-2* have a half-life of 2.5-3 hours and the steady-state mRNA level is transcriptionally regulated (Reed *et al.*, 1987; Seto *et al.*, 1988; Reed *et al.*, 1989). The primary species, Bcl-2α, is 6 kb in length. An alternatively spliced transcript of 2.4 kb, Bcl-2β, is also produced by termination within the 225 kb intron, but its 205 residues, 22 kDa protein product is expressed at very low levels (Tsujimoto and Croce, 1986; Negrini *et al.*, 1987) and its significance is unknown.

Bcl-2α mRNA translates into a 26 kDa protein product of 239 amino acids that lacks recognizable functional motifs or homologies to other proteins. Bcl-2 is non-glycosylated and can be phosphorylated (Alnemri *et al.*, 1992). The absence of

a signal peptide and a stretch of 19 hydrophobic residues bordered by basic amino acids at the carboxyl terminal indicate an intracellular membrane localization (Chen-Levy *et al.*, 1989). Indeed, immuno-electron microscopy and membrane import studies have confirmed Bcl-2's home on the outer mitochondrial membrane and the cytosolic faces of the nuclear envelope and the ER (Monaghan *et al.*, 1992; Nguyen *et al.*, 1993; Krajewski *et al.*, 1993; Lithgow *et al.*, 1994; Akao *et al.*, 1994). The patchy distribution pattern of Bcl-2 on the nuclear envelope and the outer mitochondrial membrane is reminiscent of nuclear pore complexes and mitochondrial junction complexes. Thus Bcl-2 might associate or be part of this complex (Krajewski *et al.*, 1993; de Jong *et al.*, 1994).

Anti-apoptotic activity of Bcl-2

The first report on the anti-apoptotic activity of Bcl-2 came in 1988. Vaux *et al.* has shown that Bcl-2 can protect a IL-3-dependent pre-B cell line from apoptosis following cytokine withdrawal without cell proliferation. The same survival effects are also observed in other hematopoietic cell lines dependent on growth factors such as IL-4, IL-7, and GM-CSF, but not IL-2 or IL-6 (Nunez *et al.*, 1990). Neurons are also protected by Bcl-2 upon withdrawal of nerve growth factor (Garcia *et al.*, 1992). To date, there is an impressive list of apoptosis-causing agents against which Bcl-2 can confer resistance. These include chemotherapeutic drugs (Miyashita and Reed, 1993; Kamesaki *et al.*, 1993; Walton *et al.*, 1993), Fas engagement (Itoh *et al.*, 1991), gene products such as Myc (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992) and p53 (Chiou *et al.*, 1994), heat shock (Lotem and Sachs, 1993), some viruses (Levine *et al.*, 1993; Olsen *et al.*, 1996), free radicals (Krajewski *et al.*, 1993; Hengartner and Horvitz, 1994; Kamada *et al.*, 1995), glucocorticoids, Ca^{2+}, phorbol esters, and gamma- and UV-radiation (Tsujimoto, 1989; Sentman *et al.*,1991; Strasser *et al.*, 1991).

Not surprisingly, Bcl-2 also has anti-apoptotic activity against TNF-α. Bcl-2 was identified by a cDNA expression cloning strategy to completely inhibit TNF-α-induced cell death in MCF7 breast carcinoma cells (Jäättelä *et al.*,1995). Another cell type, SK-N-MC human neuroblastoma cells, following differentiation to a neuronal phenotype, exhibit dose-dependent apoptosis upon TNF-α exposure in a Bcl-2 and CrmA suppressible manner (Talley *et al.*, 1995). This parallels caspases being TNF-α's effectors in apoptosis and points to the possibility that Bcl-2 might protect against TNF-α by inhibiting caspases. Indeed, a study by Ibrado *et al.* (1996) has demonstrated that overexpressed Bcl-2 blocks the cleavage and activation of CPP32 brought on by Ara-C (an anti-leukemic drug)-induced apoptosis in HL-60 cells, a human acute myeloid leukemic cell line. TNF-α-induced apoptosis in the same cell line is accompanied by a concomitant decrease in Bcl-2 mRNA levels (Chen *et al.*, 1995). Another line of evidence also ties Bcl-2 to TNF-α-induced apoptosis. Klefstrom *et al.* (1994) correlated transformed and tumor cell sensitivity to TNF-α cytotoxicity with dysregulated c-myc expression. Both this group and Jänicke *et al.* (1994) showed that c-myc antisense oligodeoxynucleotides and cDNA fragments can bestow resistance to TNF-α-sensitive cell lines. This TNF-α and c-myc mediated apoptosis is blocked by Bcl-2 overexpression, a finding also verified earlier by Bissonnette *et al.* (1992) and Fanidi *et al.* (1992).

There are reports that Bcl-2 does not protect against TNF-α-induced apoptosis (Vanhaesebroeck *et al.*, 1993). However, the most recent evidence strongly supports a tie between TNF-α and Bcl-2.

Bcl-2 family of cell-fate regulators

The discovery of Bcl-2 and its anti-apoptotic function has sparked intense interest in the topic, including the search for other proteins similar to Bcl-2. Based

on sequence homologies and physical interactions, a family of proteins with Bcl-2 homology has been identified. Interestingly, the family includes both members that protect against, and accelerate, apoptosis. Besides Bcl-2 itself, the protective members are Bcl-x_L (Boise *et al.*, 1993), Mcl-1 (Kozopas *et al.*,1993), A1 (Lin *et al.*, 1993), the *C. elegans* protein CED-9 (Hengartner *et al.*, 1992; Hengartner and Horvitz, 1994), and the viral homologues BHRF-1 of Epstein-Barr virus (Cleary *et al.*, 1986), LMW5-HL of African swine fever virus (Neilan *et al.*, 1993), and E1B-19K of adenovirus (Rao *et al.*, 1992; White *et al.*, 1992; Boyd *et al.*, 1994; Chiou *et al.*, 1994). The pro-apoptotic members include Bax (Oltvai *et al.*, 1993), Bcl-x_S (Boise *et al.*, 1993), Bad (Yang *et al.*, 1995), Bik (Boyd *et al.*, 1995), and Bak (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995). Bcl-x_L (long) and Bcl-x_S (short) arise from the same transcript through alternative splicing, indicating the regions that are spliced out of Bcl-x_S are important for anti-apoptotic function (see below). The fact that a Bcl-2 homologue is also found in *C. elegans* testifies to the evolutionary importance and conservation of PCD. Virus acquisition of Bcl-2 homologues into the limited space of their genome marks the survival advantage these proteins confer. Table 1.2 summarizes the members of the Bcl-2 family.

There are extensive interactions between Bcl-2 family members. For instance, Bax and Bak can dimerize with each other and with either Bcl-2 or Bcl-x_L, suggesting Bcl-2 and Bcl-x_L protect against apoptosis by complexing with and hindering the pro-apoptotic function of Bax and Bak, and *vice versa* (Sato *et al.*, 1994; Sedlak *et al.*, 1995; Farrow *et al.*, 1995). The implication is that the ratio between the amount of anti-apoptotic and pro-apoptotic proteins determines cell fate. Bcl-2 can also homodimerize. However, a mutant that can homodimerize yet not heterodimerize with Bax lacks anti-apoptotic activity, again suggesting that binding to Bax is needed to protect cells from cell death (Yin *et al.*, 1994). Bcl-x_S

can bind to both Bcl-x_L and Bcl-2. Presumably this promotes apoptosis by displacing pro-apoptotic proteins such as Bax from protective association with Bcl-x_L and Bcl-2 (Boise *et al.*, 1993). BAD operates in the same manner (Yang *et al.*, 1995). Therefore, the death promoters could function in two ways: either they interfere with the survival proteins, or the death protein dimers are directly harmful. It should be noted that although the terms homodimer and heterodimer are used, the stoichiometry of these associations is unknown.

Bcl-2 homology domains

The sequence alignment of the Bcl-2 family members identifies four conserved regions. These are called Bcl-2 Homology Domains 1, 2, 3, and 4 (BH1, BH2, BH3, and BH4), a terminology originally suggested by Oltvai *et al.* (1993). For human Bcl-2, BH1 consists of residues 136-155, BH2 of residues 187-202, BH3 residues 93-107, and BH4 10-30. BH1 and BH2 are involved in heterodimerization of Bcl-2 with Bax (Hanada *et al.*, 1995). BH4 is responsible for Bcl-2 homodimerization in a head-to-tail manner by interacting with BH1 and BH2. BH4 is also found in all anti-apoptotic members, but not in most pro-apoptotic proteins, thus indicating that it is particularly important in anti-apoptotic function. The only pro-apoptotic protein that contains BH4 is Bcl-x_S. As mentioned before, this protein arises from an alternatively spliced transcript of Bcl-x. The resulting Bcl-x_S lacks both BH1 and BH2, but retains BH3 and BH4. Therefore it probably promotes apoptosis by binding with Bcl-2 and Bcl-x_L via BH4 and sequestering them from Bax or Bak (Boise *et al.*, 1993). Bik only contains BH3, implying its significance in promoting apoptosis. This is confirmed by deletional analysis of Bax and Bak, which also contain BH3 (Chittenden *et al.*, 1995 a; Zha *et al.*, 1996). Although BH3 does exist in Bcl-2, Bcl-x_L, and Mcl-1, its sequence is less homologous with the pro-apoptotic BH3. Remarkably, substituting

Bcl-2's BH3 with that of Bax converts Bcl-2 into a cell death promoter (Hunter and Parslow, 1996).

Biochemical function of Bcl-2

There have been many proposed functions of Bcl-2 proteins. Firstly, a considerable number of studies point to an anti-oxidant function for Bcl-2, particularly control of reactive oxygen species (ROS) levels and protection from lipid peroxidation (Kane *et al.*, 1993; Hockenberry *et al.*, 1993), although this is controversial (Jacobson and Raff, 1995; Shimizu *et al.*, 1995). Since apoptosis could take place in nearly anaerobic conditions, oxidative stress is most likely the result rather than the cause of apoptosis. Secondly, Bcl-2 could be involved in intracellular Ca^{2+} homeostasis, since an early event of apoptosis is loss of Ca^{2+} from the ER. Bcl-2 overexpression prevents this loss and the accumulation of Ca^{2+} in the mitochondria (Baffy *et al.*, 1993; Lam *et al.*, 1994). This function also corresponds with the localization of Bcl-2 in intracellular membranes. The three-dimensional structure of Bcl-x_L has recently been solved. It reveals the protein to be strikingly similar to the pore-forming domains of diphtheria toxin and bacterial colicins which form ion or protein channels (Muchmore *et al*, 1996). Both Bcl-x_L and Bcl-2 have been shown to form ion channels *in vitro* (Minn *et al.*, 1997; Schendel *et al.*, 1997), but the number of molecules forming each channel is unknown. In contrast, Bcl-2 can inhibit the release of the caspase-activating protein cytochrome c from the intermitochondrial space into the cytoplasm (Yang *et al.*, 1997; Kluck *et al.*, 1997). It has not been determined whether Bax or Bak form channels that facilitate such release and whether Bcl-2 can block such channels.

Another well studied function of Bcl-2 is its ability to form complexes with many different proteins. Being an integral membrane protein oriented towards the

cytoplasm, it may serve as a dock for cytoplasmic proteins and either sequester and inactivate them, or target them for interactions with other membrane-associated proteins (Reed, 1997). Of fundamental importance is the recent demonstration that CED-9 and Bcl-x_L associate with CED-3, ICE, and FLICE, using CED-4 (a *C. elegans* pro-apoptotic protein which functions with CED-3 in causing apoptosis [Yuan and Horvitz, 1990]) as an adaptor (Spector *et al.*, 1997; Chinnaiyan *et al.*, 1997; Wu *et al.*, 1997). CED-4, normally found in the cytoplasm, is sequestered to the intracellular membranes by CED-9 and Bcl-x_L, along with the caspases, where presumably the cell death inducing enzymes can no longer act on their cytoplasmic substrates (Wu *et al.*, 1997). Alternatively, association with CED-4 and sequestration may hinder the maturation of caspases since Bcl-2 overexpression has been shown to prevent the proteolytic processing and activation of CPP32 (Chinnaiyan *et al.*, 1996; Boulakia *et al.*, 1996). Recently, Zou *et al.* (1997) have identified three proteins, named apoptosis-activating factors (Apaf 1-3), that can activate CPP32. Sequence analysis have shown that Apaf-1 is the mammalian homologue of CED-4, Apaf-2 is cytochrome c, and Apaf-3 is a 45 kDa protein that has not been cloned yet. Reconstitution of purified cytochrome c, ATP, Apaf-1, and Apaf-3 allows the proteolytic processing and activation of CPP32. Cytochrome c has also been shown to associate with Apaf-1, accounting for earlier findings that its release from the intermitochondrial space can activate apoptosis (Yang *et al.*, 1997; Kluck *et al.*, 1997). While it is known that Bcl-2 blocks cytochrome c release, it remains to be seen whether it can also bind to Apaf-1. Conversely, proteins such as Bax and Bak may promote apoptosis by binding to Bcl-2 and homologues, displacing Apaf-1 and caspases into the cytoplasm.

Another protein that forms complexes with Bcl-2 is, the serine kinase Raf-1 (Wang *et al.*, 1996). This association targets Raf-1 to the mitochondria where it phosphorylates the pro-apoptotic protein BAD. Phosphorylated BAD dissociates

from Bcl-2 and Bcl-x_L into the cytosol, where it presumably cannot interfere with the function of Bcl-2 and Bcl-x_L (Zha *et al.*, 1996 a). The region on Bcl-2 responsible for Raf-1 binding is BH4. As mentioned earlier, BH4 is found in all anti-apoptotic members, but not in most pro-apoptotic proteins. This suggests that the anti-apoptotic members evolved an additional domain for communicating with Raf-1 (Reed, 1997). Another Bcl-2-associating protein is BAG-1. It has anti-apoptotic activity (Takayama *et al.*, 1995), and collaborates with Bcl-2 by activating Raf-1 localized to the mitochondria (Wang *et al.*, 1996 a). Bcl-2 also protects cells by binding and sequestering R-ras p23, which promotes cell death caused by growth factor deprivation (Fernandez-Sarbia and Bischoff, 1993; Wang *et al.*, 1995 a). In accord with the earlier proposal that Bcl-2 can prevent calcium-mediated apoptosis, calcineurin, a Ca^{2+}-dependent protein phosphatase, induces apoptosis in a Bcl-2 suppressible manner (Shibasaki and McKeon, 1995), conceivably through dephosphorylation of BAD (Reed, 1997). Last but not least, Bcl-2 can form complexes with p53-BP2, a p53 binding protein. This interaction may explain Bcl-2 interference of p53 translocation into the nucleus and suppression of p53-dependent apoptosis (Shaw *et al.*, 1992; Ryan *et al.*, 1994; Naumovski and Cleary, 1996).

Although the many ways by which the Bcl-2 family of proteins function are becoming clear, how they fit together into a unified mechanism of survival and cell death remains enigmatic.

Expression of Bcl-2

In adults, Bcl-2 expression is restricted to tissues that are regenerated from stem cells, possess proliferative ability, or are long-lived (Krajewski *et al.*, 1994). These include epithelial cells and hematopoietic cells. For example, the regenerative basal crypt cells of the intestines express high levels of Bcl-2,

possibly to keep them from going into senescence, thus maintaining the stem cell pool (Hockenberry *et al.*, 1991).

In contrast, Bcl-2 expression is found in most fetal tissues, including hematolymphoid, epithelial, neural, endocrine, and mesenchymal cell types (LeBrun *et al.*, 1993; Lu *et al.*, 1993; Novack and Korsmeyer, 1994). Its presence in areas characterized by inductive interactions points to a role in morphogenesis, possibly involved in condensation of cells committed to forming differentiated structures such as hair follicles and kidney glomeruli (LeBrun *et al.*, 1993). Bcl-2 expression is also observed in tissues with proliferative cells and cells undergoing morphological passage from undifferentiated stem cells to committed precursor cells. One of these tissues is the placenta. LeBrun *et al.* (1993) have found placental trophoblasts to express the highest Bcl-2 levels of all the embryonic tissues examined.

1.3 THE PLACENTA

The placenta is an extra-embryonic organ that anchors the fetus to the maternal uterine wall. It is an immunological barrier against maternal allogeneic response and pathogens from the maternal circulation. But most importantly, the placenta provides the maternal-fetal interface with nutrients, water and gaseous exchanges, and other synthetic, secretory, excretory and transport functions fundamental to the maintenance of pregnancy. It undergoes synchronized structural reorganization and functional specialization throughout fetal development to accommodate the changing needs of the fetus (Ohlsson, 1989).

The fertilized embryo undergoes cell divisions and forms a blastocyst surrounded by an outer layer of cells called trophoblasts. This then attaches to the maternal uterine wall during implantation (Boyd and Hamilton, 1970). An invasive

process ensues which penetrates maternal spiral arteries. The result is the establishment of the hemochorial type of placentation where maternal blood flow comes into direct contact with fetal trophoblasts (Aplin, 1991; Strickland and Richards, 1992). The blastocystic trophoblasts then differentiate and give rise to the anchoring villi, floating villi, and amniotic membrane trophoblasts (Damsky *et al.*, 1992).

The anchoring villi attach the fetus to the uterine wall by embedding into the decidualized maternal endometrium. Trophoblasts below the anchoring villi are known as extravillous trophoblasts. They are non-polarized and are organized into multilayered columns penetrating deep into the endometrium and ultimately displacing the endothelium of the spiral arteries (Damsky *et al.*, 1992). The outcome is facilitation of maternal blood flow into the intervillous spaces.

Most of the metabolic processes that support fetal growth occur in the floating villi trophoblasts. These are polarized cells that do not contact the endometrium (Benirschke and Kaufmann, 1990). Placental villi are enclosed by a trophoblast layer consisting of a continuous syncytium made up of multinucleated syncytiotrophoblasts (ST), which face maternal blood, and an underlying layer of mononucleated stem cell trophoblasts called cytotrophoblasts (CT), that continuously proliferate and fuse with the syncytium. Proliferation occurs in the first and second trimester of pregnancy only and fusion occurs in all three trimesters. A basement membrane separates the trophoblasts from the villous stroma. Within the villous stroma lie fetal capillaries and stromal cell such as fibroblasts, fetal macrophages, and a few mast cells (Fox, 1978). Figure 1.1 is a schematic representation of the floating and anchoring villi.

The trophoblasts

The primary function of cytotrophoblasts is to proliferate and provide cells for the overlying syncytium. They have a large nucleus, prominent nucleoli, numerous free ribosomes, and poorly developed ER and Golgi apparatus characteristic of undifferentiated cells (Contractor *et al.*, 1977). Syncytium is formed by the fusion of CTs, as shown by time-lapse cinematography. CTs spontaneously aggregating and fusing to form syncytium in culture (Kliman *et al.*, 1986). Syncytialization can occur in basal medium, but its supplementation with growth factors such as epidermal growth factor (EGF) accelerates the process (Morrish *et al.*, 1987 a; Barnea *et al.*, 1990). A phenotype common to most STs is prevailing rough ER and extensive microvilli on the cell surface. This facilitates active transfer, metabolism, and peptide hormone synthesis (Morrish *et al.*, 1987). A minor ST phenotype is prevalent in smooth ER specialized in steroid hormone metabolism (Boyd and Hamilton, 1970). Importantly, both ST and CT are devoid of class I HLA molecules, partly accounting for the absence of maternal allogeneic responses against the fetus.

A major obstacle in the study of trophoblasts has been contamination of cultures with cells from the villous stroma such as fibroblasts. If present, these cells would eventually overgrow the culture, skewing biochemical analyses. One feature of trophoblasts is the absence of the CD9 antigen on their cell surface (Morrish *et al.*, 1991). This finding has led Yui *et al.* (1994 a) to develop a method for the purification of trophoblasts using column-immunoelimination of CD9-expressing cells such as fibroblasts. In this method, fresh tissue from term placenta is digested with trypsin into a single cell suspension, which is then incubated with anti-CD9-antibody before being passed through an anti-Ig-antibody-conjugated glass bead column to remove contaminating cells. This method routinely yields primary trophoblast cultures of more than 99.98% purity that can survive for up to

three weeks. These cells can be induced to form syncytium in the presence of EGF. For the rest of this thesis, CT and ST refers only to villous trophoblasts.

Apoptosis of the trophoblasts

TNF-α and IFN-γ (interferon gamma) receptors are expressed on the surface of ST and CTs (Peyman and Hammond, 1992; Valente *et al.*, 1992; Yelavarthi and Hunt, 1993), but cellular responses have been mostly detrimental. For example in mice, higher levels of TNF-α and IFN-γ mRNAs are found in placental tissues from mating combinations with high rates of spontaneous fetal resorption compared to normal pregnancies (Tangri and Ragupathy, 1993). Secondly, injections of TNF-α into pregnant rats (Silen *et al.*, 1989), and TNF-α or IFN-γ into pregnant mice (Chaouat *et al.*, 1990), result in placental necrosis and fetal demise. Thirdly, placental and decidual TNF-α production brought on by local microbial infections have been implicated in the pathogenesis of pre-term labor (Casey *et al.*, 1989; Romero *et al.*, 1989; Hillier *et al.*, 1993). Lastly, IUGR (intrauterine growth retardation) fetuses have elevated TNF-α in their amniotic fluid (Heyborne *et al.*, 1992). The mechanism for the detrimental effects of TNF-α and IFN-γ on placenta and fetal development is not well understood. One possibility is that TNF-α induces apoptosis in both CT and ST thereby compromising placental functions and integrity. Yui *et al.* (1994 b) found that TNF-α created holes in cultured cell monolayers by inducing trophoblast apoptosis. IFN-γ alone does not cause apoptosis, instead it synergizes with TNF-α to enhance the cytotoxic effect, yielding 40-60% lethality.

However, the presence of TNF-α does not always correlate with detrimental effects. TNF-α is also expressed in normal, healthy utero-placental tissues (Bulmer *et al.*, 1990; Chen *et al.*, 1991; Paulesu *et al.*, 1994), suggesting a physiological

role for TNF-α during normal placental development. Substantial histological evidence exists for trophoblast turnover (Parmley 1990; Simpson *et al.*, 1992) since, like other organs, placental development and tissue maintenance depend on the balance between cell proliferation, maturation, and death. TNF-α thus may regulate trophoblast growth and placental villi tissue remodeling due to its apoptosis-inducing effects. On the other side of the balance lies tissue renewal, accomplished by positive regulators of trophoblast growth. One such positive regulator is EGF, an abundant hormone in maternal and fetal serum, and in amniotic fluid (Adamson, 1990). It influences various trophoblast biological functions including migration (Bass *et al.*, 1994), proliferation (Maruo *et al.*, 1992), morphological differentiation, and peptide hormone secretion (Morrish *et al.*, 1987). There is also a strong correlation between alteration in placental EGF receptor expression with IUGR (Fondacci *et al.*, 1994). Perhaps one of the most important insights into the effects of EGF on placenta is the finding that it can antagonize TNF-α/IFN-γ-induced apoptosis in cultured CTs and ST from term placentas (Garcia-Lloret *et al.*, 1996). This finding paints an interesting picture of the role of cytokines in trophoblast biology. The balance between cell growth and death can be achieved by coordinated interplay of positive and negative regulators. On one hand the placenta uses TNF-α/IFN-γ to regulate cell growth and remodel tissue, while on the other hand it uses EGF to protect against excessive and untimely TNF-α/IFN-γ-induced cytotoxicity and possibly repair damaged tissues by inciting fusion of the stem cell reserve of CTs with the syncytium (Yui, 1994).

Mechanisms of protection against trophoblast apoptosis

The mechanism of EGF protection against TNF-α/IFN-γ-induced apoptosis is unknown. EGF could induce a set of genes in the trophoblast that can block the signal transduction of TNF-α or activates inhibitory signals. An obvious candidate

is Bcl-2 because of its well documented role in embryogenesis (Baer, 1994) and protection against TNF-α-induced apoptosis via the caspase pathway. There have been very few studies on Bcl-2 expression in the trophoblasts in human, and those in the literature largely contradict each other. Within the placenta, Bcl-2 expression is reportedly restricted to trophoblasts (LeBrun *et al.*, 1993). However, Kim *et al.* (1995) have detected the protein in other villous stromal cell types. LeBrun *et al.* (1993) have also found that both CTs and ST stained equally intensely for Bcl-2, while Lu *et al.* (1993) have found Bcl-2 expressed only in ST but not CTs. Adding to these conflicting findings is the observation that Bcl-2 protein levels decline after 33 weeks of gestation and thereafter remain undetectable by immunoblotting (Kim *et al.*, 1995). Few reports have been made concerning the effects of EGF onthe expression of Bcl-2 family members with the exception of Nass *et al.* (1996). These investigators show that apoptosis in epithelial cell lines (derived from MMTV-*myc* mammary tumors) brought on by the withdrawal of EGF is correlated with a drop in Bcl-x_L expression. However, Bcl-x_L expression is high in the presence of EGF.

The conflicting results and the lack of reports on this topic warrant further detailed examinations to understand the role of Bcl-2 in the placenta. I therefore set out to study Bcl-2 expression in the trophoblasts and its significance with respect to EGF protection from TNF-α/IFN-γ-induced apoptosis.

Table 1.1 Differences between apoptosis and necrosis

Features	Apoptosis	Necrosis
tissue distribution	single cells	groups of cells
tissue reaction	phagocytosis	cellular exudate
morphology	cell shrinkage	cell swelling
	organelles intact	organelles damaged
	chromatin marginated, condensed	chromatin fragmented
	cytoplasmic extensions	cytoplasm fragmented
	membrane intact	membrane damaged

Adapted from Bosman, Visser, and van Oeveren, 1996

Table 1.2 Bcl-2 family of cell fate regulators

Gene product	Function
Bcl-2	inhibitor of apoptosis, binds Bax and Bak
Bcl-x_L	inhibitor of apoptosis, binds Bax and Bak
Mcl-1	inhibitor of apoptosis
A1	sequence homology with Bcl-2
CED-9	*C. elegans* cell death inhibitor
ASFV LMW5-HL	African swine fever virus Bcl-2 homologue
EBV BHRF-1	Epstein-Barr virus inhibitor of apoptosis
E1B 19K	adenovirus inhibitor of apoptosis, binds Bax and Bak
Bax	accelerator of apoptosis, binds Bcl-2, Bcl-x_L, and E1B 19K
Bak	accelerator of apoptosis, can also be inhibitor, binds Bcl-2, Bcl-x_L, and E1B 19K
BAD	accelerator of apoptosis, binds Bcl-2 and Bcl-x_L
Bik	accelerator of apoptosis, has only BH3 domain, binds Bcl-2, Bcl-x_L, E1B 19K, and BHRF-1

Adapted from White, 1996

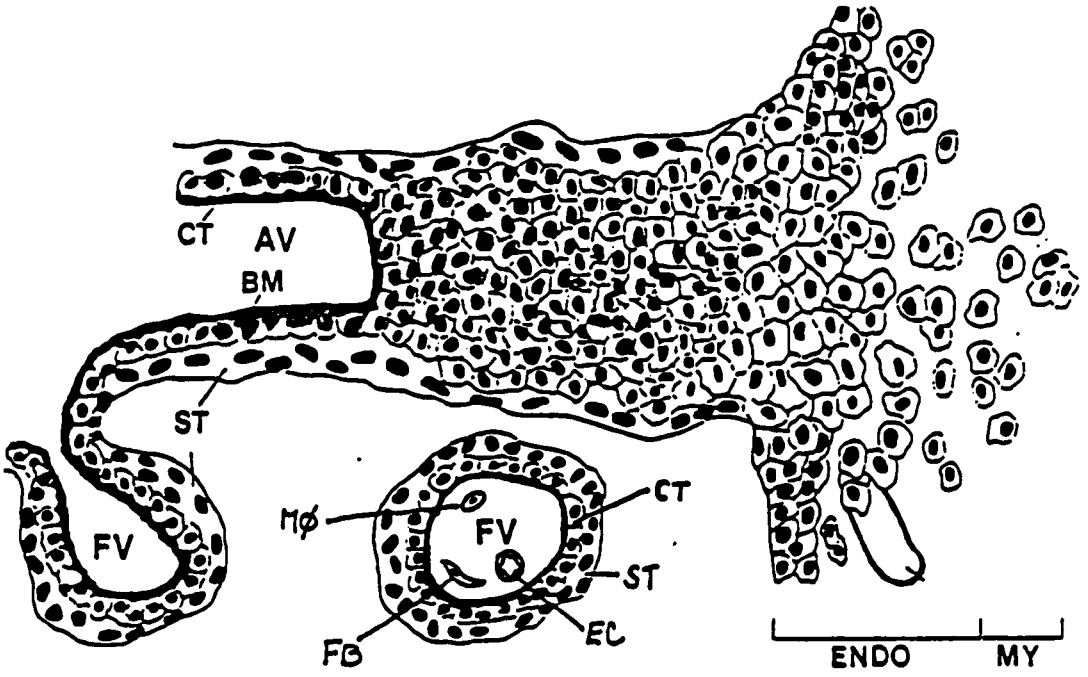


Figure 1.1 Schematic representation of the fetal-maternal interface. A floating villus (FV), anchoring villus (AV) with an associated cell column, and the maternal uterine wall are shown. In the FV, cytotrophoblasts (CT), separated from the stromal core by a basement membrane (BM), fuse to form the overlying syncytiotrophoblasts (ST) whose apical surface is bathed in maternal blood. Within the stromal core are fibroblasts (FB), macrophages (MΦ), and fetal endothelial cells (EC). In the AV, extravillous trophoblasts proliferate and form a cell column which penetrates the maternal endometrium (ENDO) and myometrium (MY), thus anchoring the placenta to the uterine wall.

Adapted from Damsky et al., 1989

CHAPTER 2

HYPOTHESIS, RATIONALE, AND EXPERIMENTAL APPROACH

2.1 HYPOTHESIS

As mentioned in the introduction, together with cell proliferation, programmed cell death is an integral element of tissue homeostasis. The process is controlled by two sets of interacting genes: one mediates cell death, while the other mediates cell survival. Cytokines play the pivotal role in modulating placental growth and development. Of particular interest is the observation that TNF-α/IFN-γ mediates apoptosis in the two types of trophoblasts, CTs and ST, and EGF antagonizes this effect. The mechanism of EGF antagonism is unknown, but the anti-apoptotic protein Bcl-2 is a logical intermediate in the process. ***Therefore I hypothesize that TNF-α/IFN-γ-induced apoptosis in placental trophoblasts correlates with down-regulation of Bcl-2, while EGF confers resistance against such apoptosis through up-regulation of Bcl-2.***

2.2 RATIONALE

There is extensive evidence in the literature on the pathway that TNF-α uses to trigger apoptosis. Namely, TNF-α binding to its receptor activates downstream caspases, which execute the cell death program. Their substrates include proteins that are crucial to the maintenance of the cell's well being. Conversely, Bcl-2 is a well-documented antagonist against TNF-α-mediated apoptosis. Mechanistically, the most studied pathway by which Bcl-2 affords protection to cells is through its ability to inhibit caspase activities.

The discovery that TNF-α induces PCD in placental trophoblasts and that EGF opposes such cell death prompted me to envision Bcl-2 as an EGF-response gene, probably controlling TNF-α-induced apoptosis via inactivation of caspases within trophoblasts. The proposal is that, in trophoblasts committed to apoptosis,

Bcl-2 is down-regulated at the protein level in order for the cell death program to proceed. In an opposite manner, EGF could up-regulate Bcl-2 to counteract the harmful effect of TNF-α. This is an important topic of study because of the strong correlation between TNF-α and such complications of pregnancy as IUGR, spontaneous fetal resorption, and fetal infection due to the breakdown of the fetal-maternal barrier. Furthermore, the positive effect that EGF has on the placenta and fetal development is well-documented but not completely understood. Could Bcl-2 be the answer? Could TNF-α-related complications during pregnancy be rectified with the administration of EGF? Because of the lack of studies on the expression and role of Bcl-2 in trophoblasts, and the contradictory results of existing research, I have examined this issue and attempted to clarify the role of Bcl-2 in trophoblasts. This laboratory is uniquely equipped for the pursuit of just such a study by virtue of the ability to obtain, purify, and culture human primary trophoblasts that have been well characterized (Yui *et al.*, 1994 a). This *in vitro* culture system offers a close approximation of the *in vivo* situation. Studies on trophoblasts, and those on Bcl-2's role in cell death in general, are primarily performed with cell lines. The cell line model for trophoblasts are choriocarcinoma cell lines that generally do not behave like villous trophoblasts (for example they proliferate and express class I HLA molecules [Kovats *et al.*, 1990; Hunt and Orr, 1992], while primary trophoblasts do not). When primary trophoblasts are studied, the cultures are usually contaminated with other cell types, skewing the analyses. Our culture system routinely yields trophoblast preparations that are >99.98% pure. Moreover, CTs can be driven to differentiate into ST by co-culturing with EGF, allowing the examination of both cell types. TNF-α is always utilized in conjunction with IFN-γ throughout this project to achieve maximum effects on the cells.

2.3 EXPERIMENTAL APPROACH

The expression of Bcl-2 in the placenta is first examined by staining for the protein immunohistochemically in tissue sections from first trimester and term placentas. This is followed by a study of Bcl-2 expression in cultured first trimester and term CT and ST Bcl-2 expression in culture using immunoblotting and immunohistochemical staining. Flow cytometric analysis is also performed to probe any heterogeneity in Bcl-2 level in a population. These results clarify the expression of Bcl-2 in trophoblasts and are presented in Section 4.1 of Chapter 4. Section 4.2 recounts the finding of TNF-α/IFN-γ-mediated apoptosis in trophoblast and compares Bcl-2 levels of trophoblasts cultured in different cytokines in order to test the hypothesis. In Section 4.3 flow cytometry is used as a tool to correlate the low level of Bcl-2 with predisposition to TNF-α/IFN-γ-induced apoptosis. Finally in Section 4.4 an attempt is made at transfecting trophoblasts with an Bcl-2 expression vector to test its protective capability.

CHAPTER 3

MATERIALS AND METHODS

3.1 CELL CULTURES AND TISSUE SECTIONS

Isolation and purification of human term placental trophoblasts

Human term and first trimester placentas from spontaneous vaginal deliveries or elective cesarean sections were used as sources for trophoblasts. Suspensions of placental cells were prepared from chorionic villous tissues by using trypsin-DNase digestion as described by Morrish *et al.* (1987). The removal of red blood cells and the subsequent purification of trophoblasts with the use of Ig-conjugated glass bead columns (Biotex, Edmonton, AB) to eliminate CD9-positive mesenchymal cells has been described in detail by Yui *et al.* (1994 a). Purified cells were routinely cryopreserved in 10% (v/v) dimethylsulfoxide under liquid nitrogen.

Culturing of human term placental trophoblasts

After being thawed rapidly in a 37°C water bath, cells were washed once with warm Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY) supplemented with 0.5% (v/v) 60°C heat-inactivated fetal calf serum (FCS; Gibco) and 50μg/ml gentamycin (Gibco) or 100 units penicillin/100 μg streptomycin per ml (Gibco). Following resuspension in 10% FCS/IMDM, trophoblasts were plated at $1x10^5$ cells per 100μl per well in Nunclon 96-well plates (NUNC, Roskilde, Denmark) or $1x10^6$ cells/ml per well in Linbro 6-well plates (ICN, Aurora, OH). These concentrations give a confluent monolayer when cells were optimally isolated and therefore were used throughout the experiments unless stated otherwise. After a 4 hour incubation at 37°C in 5% (v/v) CO_2 to allow adherence, non-adherent cells were removed by gently washing 5 times with warm 0.5% (v/v) FCS/IMDM. Adherent cells were maintained in 10% (v/v) FCS/IMDM in a 37°C/5% CO_2 incubator. In all experiments, cytokines were added to the

trophoblasts after the 4 hour adhesion step and non-adherent cells were washed away.

Cell line

BeWo cells, a choriocarcinoma cell line, was propagated in 15% (v/v) FCS/IMDM. They were harvested with trypsin-EDTA (Gibco) and seeded at $1x10^6$ cells per well in Linbro 12-well plates (ICN, Aurora, OH). Experiments were performed on confluent cell monolayers.

Cytokines and reagents added to cultures

Recombinant human (rHu) EGF (Pepro Tech Inc., Rocky Hill, NJ) and TNF-α (Hoffmann LaRoche, Basel, Switzerland) were used at 10 ng/ml. rHu IFN-γ (Collaborative Biochemical Products, Bedford, MA) was used at 100 U/ml. rHu GM-CSF (granulocyte-macrophage colony stimulating factor; Sandoz, Basel, Switzerland) was used at 25 ng/ml and 50 ng/ml. Cycloheximide (CHX; Sigma) was used at a sublethal dose of 300 ng/ml. Dexamethasone (Dex; Sigma) was dissolved in ethanol and used at 10 μg/ml and 20 μg/ml.

Preparation of tissue sections from first trimester and term placenta

Preparation of placental sections was performed by Bonnie Lowen and Roger Miller of this laboratory. Briefly, placental tissues were fixed in 10% (w/v) buffered formalin overnight and embedded in paraffin. 4 μm sections were cut and adhered to slides pretreated with 2% (w/v) APTEX coating.

Deparaffinization of tissue section

Paraffin-embedded, fixed, placental villi sections were washed two times in xylene for 10 minutes with gentle shaking, followed by two washes with absolute ethanol for 4 minutes and two quick washes with deionized-distilled water (DDW).

Target retrieval of deparaffinized tissue sections

Deparaffinized tissue sections were placed in Target Retrieval Solution (TRS; Dako, Carpinteria, CA) in 100°C water bath for 20 minutes to allow renaturation of proteins. The TRS with the sections was then allowed to cool down at room temperature (RT) for 20 minutes. After cooling, the sections were washed in Wash Buffer (WB; per liter contains 1.39 g Tris-base, 6.06 g Tris-HCl, 17.53 g NaCl, and 1 ml Tween-20) for 5 minutes. The sections were now ready for immunohistochemical staining.

3.2 IMMUNOHISTOCHEMISTRY

Immunohistochemical staining of tissue sections

Deparaffinized, renatured tissue sections were incubated with 100 µl 3% (v/v) H_2O_2 at RT for 15 minutes to quench endogenous peroxidase activities. Upon removal of this solution, non-specific Ig binding sites were blocked with 50 µl 10% (v/v) normal rabbit serum (Zymed, San Francisco, CA) for 1 hour. Leaving the blocker on the sections, 5 drops of either optimally diluted mouse anti-human Bcl-2 antibody or mouse IgG1 isotype control (Dako) were added and left in 4°C overnight. The antibodies were removed with 1 quick rinse and 2 more washes in WB for 5 minutes. Biotinylated rabbit anti-mouse second antibody (Zymed) was added for 1 hour, followed by 1 quick rinse and 1 five-minute wash with WB. An amplifying agent consisting of streptavidin and biotinylated horse radish

peroxidase (HRP; Dako) was added for 15 minutes. The sections were then washed with 1 quick rinse and 1 five-minute wash with WB, followed by 1 quick wash with DDW. Color development was achieved by AEC substrate/chromogen bulk kit (Dimension, Mississauga, ON). Positive staining yielded a red color. The sections were counterstained with hematoxylin (diluted 1:1 with DDW; Sigma, St. Louis, MO), rinsed in DDW, and dipped in 37 mM ammonia for bluing of the background (to provide contrast with the red positive staining). The sections were then mounted with GVA Mount (Zymed). Photographs were taken with a Zeiss photomicroscope.

Immunohistochemical staining of cultured trophoblasts

The staining was performed according to the directions supplied by the Histostain Kits purchased from Zymed. Trophoblasts cultured in Nunclon 96-well plates were washed once with phosphate buffered saline (PBS) upon removal of culture medium and fixed in methanol at -20°C for 10 minutes. The methanol was then removed and the fixed cells were washed with PBS 3 times, quenched in 3% H_2O_2 at RT for 15 minutes, and blocked for 1 hour with non-immune goat serum supplied with the kit. After removing the blocker, 30 µl of the following antibodies were added: mouse monoclonal anti-human Bcl-2 (20 µg/ml; CalBiochem, San Diego, CA); mouse monoclonal anti-human desmosomal protein (1:100; Sigma); and mouse IgG1 isotype control (20 µg/ml for Bcl-2 staining control; 1:100 for desmosome staining control; Dako). The plates were sealed with parafilm and incubated at 4°C overnight. The wells were washed rapidly 2 times with PBS and twice more with 10-minute incubations on a plate shaker. Biotinylated goat anti-mouse IgG from the kit was added for 30 minutes at RT, and washed as above. The streptavidin-peroxidase conjugate from the kit was added for 15 minutes at RT, and washed as above. One more quick wash in DDW preceded the color

development which was achieved by AEC substrate/chromogen bulk kit (Dimension). Positive staining yielded a red color. Cells were counterstained with hematoxylin in the case of desmosomal staining to identify nuclei. Photographs were taken with a Telaval 31 inverted photomicroscope.

3.3 FLOW CYTOMETRIC ANALYSIS

Trophoblasts ($1x10^6$ cells/well) cultured in Linbro 6-well plates were detached with trypsin-EDTA (Gibco), washed once in PBS containing 1% (w/v) bovine serum albumin (BSA; fraction V; Sigma) and 0.02% (w/v) NaN_3 (PBSA). From this point on, all procedures were performed on ice. Cells were fixed in 1% (w/v) paraformaldehyde buffered to pH 7.4-7.6 for 20 minutes with frequent, vigorous mixing to prevent clumping of cells. The reaction was stopped by the addition of an equivalent volume of PBSA, centrifugation at 1500 rpm for 5 minutes, followed by one more wash with PBSA. Non-specific sites were blocked by incubating cells with 100 µl of 10% (w/v) goat serum in balanced salt solution with 0.1% (w/v) saponin (BSSS; 0.2 g/L $CaCl_2$, 0.097 g/L $MgSO_4$, 0.1% saponin, and 0.01% NaN_3 in PBS) for 30 minutes. After removing the blocker, 30 µl of either 20 µg/ml fluorescein isothiocyanate (FITC)-conjugated mouse anti-human Bcl-2 (Dako; FITC conjugation kindly performed by Dr. Linda Pilarski) or 20 µg/ml FITC-conjugated mouse IgG1 isotype control (Dako), diluted in BSSS plus 1% (v/v) goat serum, was added for 3 hours, with occasional mixing. The cells were then washed once in BSSS before leaving in BSSS overnight to allow diffusion of excess antibodies out of the cells. The cells were washed twice more in BSSS before resuspension in PBS and transfer to 12x75 mm tubes (Falcon 2058) for analysis on a FACSCAN machine using the supplier's software program LYSYS II (Benton-Dickson, San Jose, CA). When DNA content was being

studied, instead of resuspension in PBS, the cell pellet was incubated with 50 μg/ml propidium iodide in PBS for 30 minutes before analysis.

3.4 TUNEL ASSAY

This assay detects DNA fragmentation in apoptotic cells using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin DNA-nick end labeling (TUNEL; Gavrieli *et al.*, 1992). Supernatants were asperated and the cultures in 96-well plates were fixed with acetone/methanol (1:1) for 10 minutes at RT, washed 3 times with PBS, and incubated in TdT buffer (30 mM Tris-base, pH 7.2; 140 mM sodium cacodylate; 1 mM cobalt chloride) for 10 minutes. After removing the TdT buffer, 30 μl of a mixture containing 16.5 μM bio-16-dUTP (Sigma), 16.5 μM dATP (Boehringer Mannheim, Laval, Quebec), and 5 U/μl TdT enzyme (Boehringer Mannheim) in TdT buffer was added and incubated at 37°C for 2 hours. The reaction was stopped by adding 2X SSC (300 mM NaCl; 30 mM sodium citrate) for 10 minutes, followed by three 5-minute washes with DDW. Endogenous peroxidase activities were quenched in 3% H_2O_2 for 10 minutes, followed by 3 washes with DDW. PBS with 3% (w/v) skim milk and 0.5% (v/v) Tween-20 was added for 30 minutes to block non-specific sites and ExtrAvidin-HRP (Sigma) diluted 1:75 in the same blocker was added for another 30 minutes. The cells were then washed with DDW 3 times and color development was achieved by AEC substrate/chromogen bulk kit (Dimension). Positive staining yielded a dark red color. Cells were counterstained with hematoxylin. Positive controls of the reaction were generated by incubating cells in 1 μg/ml DNase I (Sigma) prior to labeling, whereas negative controls involved carrying out the TUNEL reaction in the absence of bio-16-dUTP.

3.5 GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Cell lysis

Supernatants were asperated and the cultures in 6-well plates were washed gently 3 times with PBS. 400 µl of a lysis buffer containing, in PBS, 0.5% (v/v) Nonidet-P 40 (NP40) , 14 µM leupeptin (Boehringer Mannheim), 10 µM pepstatin A (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 1 mM benzamidine (Sigma), and 0.01 mg/ml trypsin inhibitor (Sigma), was added with rocking in 4°C for 1 hour. Occasionally, cells were lysed with 1% (w/v) SDS (sodium dodecyl sulfate) in water and DNA sheared by passage through a 26 gauge needle. Lysates were collected and centrifuged at 13000 rpm, 4°C, for 5 minutes to remove non-soluble fraction. Supernatants were collected for determination of protein content. The lysates were stored in -70°C until used. When examining non-adherent cells, the supernatant was collected, spun down, and the cell pellet washed 3 times with PBS, before being subjected to lysis.

Assay for protein content

In a 96-well microtiter plate, five replicates of 10 µl of cell lysates were mixed with 150 µl BCA protein assay reaction mixture (Pierce, Rockford, IL). The plate was then incubated at 37°C for 30 minutes before absorbance was measured at 570 nm on a UVmax microplate reader (Molecular Devices). BSA was used as the protein standard.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using standard methods (Harlow and Lane, 1988). Aliquots of lysates containing

equivalent amounts of protein (usually 40-50 μg from $1x10^6$ cells) were added to ¼ volume of concentrated 4X reducing sample buffer (4XRSB; 0.25 M Tris-HCl, pH 6.8; 8% (w/v) SDS; 40% (v/v) glycerol; 2.84 M β-mercaptoethanol; 5% (w/v) bromophenol blue) and boiled for 10 minutes before loading onto a 4-12% polyacrylamide gel containing 0.1% (w/v) SDS.

Immunoblotting (western blot)

After electrophoresis, proteins were transferred electrophoretically onto a Immobilon-P PVDF membrane (Millipore, Bedford, MA). The membrane was submersed in western blot buffer (WBB; 20 mM Tris-base; 137 mM NaCl; 0.1% Tween-20) containing 5% (w/v) skim milk for at least 4 hours before incubating with 0.1 μg/ml monoclonal mouse anti-human Bcl-2 antibody (Dako) in 5% skim milk/WBB for 1 hour. The antibody solution was then decanted, the membrane quickly washed twice, followed by two more 5-minute washes, and one 15-minute wash with WBB. Secondary goat anti-mouse IgG antibody conjugated to HRP (Jackson) diluted 1:20000 with 5% skim milk/WBB was added to the membrane for 1 hour, followed by removal of the secondary antibody, two quick washes, four 5-minute washes, and one 15-minute wash with WBB. Finally, ECL reagent (Amersham) was added to the membrane for 1 minute to activate fluorescence before exposure to film (NEF-496, Du Pont, Mississauga, ON) for 5 minutes. In order to test sensitivity of this method, the dose-response relationship between the amount of protein present and the immunoblot protein band intensity was examined (Figure 3.1). Various amounts of trophoblast lysates (100, 50, and 25 μg of total cellular protein) were loaded in each lane and perform SDS-PAGE and immunoblot as deacribed above. Under these conditions, the decrease in the amount of protein present corresponded with a decrease in Bcl-2 band intensities,

indicating that this method possesses the sensitivity to detect at least two-fold changes in Bcl-2 protein levels.

3.6 CELL TRANSFECTION

Plasmids

Bcl-2 expression plasmids pR509-8-45 (sense Bcl-2) and pR509-8-18 (antisense Bcl-2) were kind gifts from Dr. John C. Reed of Burnham Institute in La Jolla, USA. Details of their construction were outlined in Reed *et al.*, 1990. Their maps were reproduced here in Figure 3.2. Briefly, the Bcl-2 cDNA pB4 was subcloned in both orientations into the 3' end of the cytomegalovirus (CMV) promoter/enhancer of the retroviral expression plasmid pBC140 to create sense and antisense Bcl-2. The pBC140 constructs contain a G418-resistance gene whose transcription was driven from the 5'-long terminal repeat (LTR). β-galactosidase expression plasmid pcDNA3/Zeo/nlacZ was a kind gift from Dr. Lung-Ji Chang of Department of MMI, University of Alberta. Its map was also reproduced in Figure 3.2. Briefly, the β-galactosidase cDNA pSP72nu-lacZ was subcloned into the 3' end of the CMV promoter/enhancer of the expression plasmid pcDNA3.1/Zeo.

Transfection

BeWo cells and trophoblasts were transfected by calcium phosphate-DNA coprecipitation (Graham and van der Eb, 1973) with slight modifications. Confluent BeWo cells (in 12-well plates) or CTs (in 6-well plates) were cultured for 20 hours prior to transfection. 2 μg of purified plasmid DNA was added in 90 μl of DDW and mixed with 10 μl of 2.5 M $CaCl_2$ in a polycarbonate tube. 100 μl of BES-buffered solution (50 mM N,N-bis(2-hydroxyethyl)-2-aminoethane-

sulfonic acid [CalBiochem], 280 mM NaCl, and 1.5 mM Na_2HPO_4 [pH 6.95]) was added dropwise and the solution is allowed to sit at RT for 45-60 minutes before the addition to 2 ml culture maintained at pH 7.1. The control consisted of cells that had been mock transfected (no plasmid DNA). Cultures were maintained in a 3% CO_2 incubator at 37°C overnight, washed with medium, and incubated in 5% CO_2 incubator for up to 5 days.

β-galactosidase staining

Following transfection and incubation, β-galactosidase over-expressing cells were stained with X-gal. Medium is removed and cells washed once with PBS before fixing with 1% (w/v) formaldehyde and 0.2% (w/v) gluteraldehyde in PBS for 5 minutes. Cells were then washed 3 times with PBS and a reaction mixture containing 4 mM potassium ferrocyanide, 4 mM potassium ferriccyanide, 2 mM $MgCl_2$, and 0.3 mg/ml X-gal (dissolved in DMF) in PBS is added for 16-20 hours at 37°C. β-galactosidase over-expressing cells were stained blue.

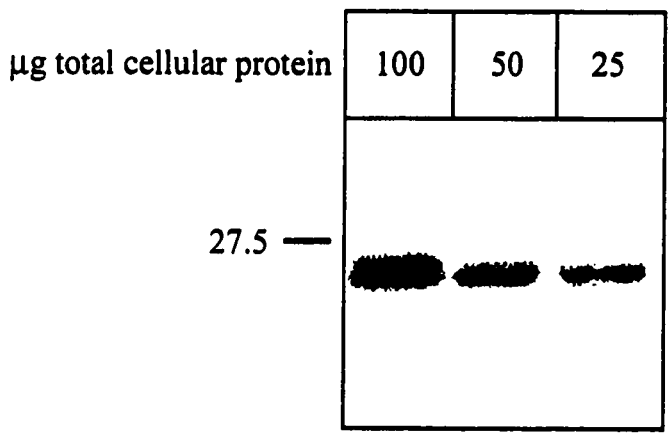


Figure 3.1 Dose-response relationship between the amount of protein and immunoblot protein band intensity. Various amounts of purified trophoblast lysates were loaded on a 4-12% gel to give the amount of total cellular protein in each lane as indicated. SDS-PAGE was performed as described in Materials and Method. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size marker in kDa is shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein.

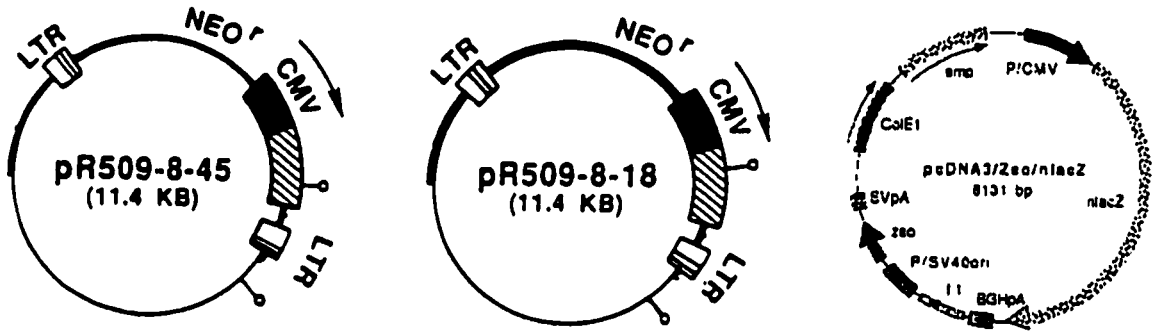


Figure 3.2 Maps of Bcl-2 and β-galactosidase expression plasmids. The Bcl-2 expression plasmids pR509-8-45 (sense Bcl-2) and pR509-8-18 (antisense Bcl-2) were kind gifts from Dr. John C. Reed. They consisted of the Bcl-2 cDNA pB4 subcloned in both orientations into the 3' end of the cytomegalovirus (CMV) promoter/enhancer in the retroviral expression plasmid pBC140. The β-galactosidase expression plasmid pcDNA3/Zeo/nlacZ was a kind gift from Dr. Lung-Ji Chang. It consisted of the β-galactosidase cDNA pSP72nu-lacZ subcloned into the 3' end of the CMV promoter/enhancer in the expression plasmid pcDNA3.1/Zeo. Maps of pR509-8-45 and pR509-8-18 reproduced from Reed et al., 1990. Map of pcDNA3/Zeo/nlacZ provided by Dr. Lung-Ji Chang.

CHAPTER 4

RESULTS

4.1 Bcl-2 EXPRESSION IN PLACENTAL TROPHOBLASTS

The few studies on the expression and role of Bcl-2 in the placenta, and in particular in placental trophoblasts, have yielded conflicting results. Therefore, experiments were first conducted to clarify *in vivo* Bcl-2 expression in the placenta.

Bcl-2 expression in first trimester and term placental villi

To study the expression of Bcl-2 protein in the placental villi, tissue sections from first trimester and term placentas were immunohistochemically stained for the Bcl-2 protein using a monoclonal mouse anti-human Bcl-2 antibody (Dako). The staining was repeated in six different batches of placental sections. As shown in Figures 4.1, Bcl-2 staining, denoted by the red color, is strong and primarily limited to the term (Figure 4.1a) and first trimester (Figure 4.1c) trophoblast layers. The staining is of approximately equal intensity between the two types of placentas (the procedural and color development times were kept constant for all sections). The syncytiotrophoblast layer is stained strongly in both cases. There are very few cytotrophoblasts in term placentas. Thus, relative ST/CT staining cannot be discerned. The intense staining of first trimester ST and the almost continuous stacking of CT make it difficult to conclusively assign relative ST/CT staining in this tissue. The stroma shows little, if any, Bcl-2 staining, and is counterstained with hematoxylin to give a blue color. The isotype control antibody stains neither the stroma nor the trophoblasts, indicating the staining of Bcl-2 is specific. Therefore, Bcl-2 is expressed in both first trimester and term villous syncytiotrophoblasts *in vivo* at approximately equal levels.

Purified first trimester and term cytotrophoblasts express the same amount of Bcl-2 *in vivo*

Using the method of Yui *et al.* (1994 a), primary cytotrophoblasts were purified from fresh placental tissue to more than 99.98% purity using elimination columns. This allows the biochemical analysis of trophoblasts without the problem of contamination with other cell types. To determine whether CTs express Bcl-2 in first trimester and term placentas and to compare relative levels in the two tissues, trophoblasts ($1x10^6$ cells) from the two types of placentas were lysed and subjected to immunoblot (western blot) analysis with anti-Bcl-2 antibody. To estimate *in vivo* levels, cells were freshly isolated, purified, and subjected to analysis. As shown in Figure 4.2, the protein level is the same in both first trimester and term trophoblasts. Therefore, the expression of Bcl-2 protein is invariant with the developmental stages of the placenta. The same result observed in cells isolated from six different placentas indicates that the expression of Bcl-2 is relatively constant among individuals.

Purified cytotrophoblasts and syncytiotrophoblasts express the same amount of Bcl-2 in culture

The immunohistochemical staining studies described above are qualitatively informative about the expression pattern of Bcl-2 in the placenta. However, they are unsuited for analyzing CTs and STs, especially at term. Immunoblot analysis on the other hand is more suited for comparing cultured CTs and STs. This can be carried out in cultures where the cell number and differentiation state are known. In order to compare CT and ST Bcl-2 expression, earlier studies on EGF stimulated differentiation of CTs to STs were repeated (Morrish *et al.*, 1987; Garcia-Lloret *et al.*, 1996). Purified cytotrophoblasts from term placenta were cultured either in medium alone or medium supplemented with 10 ng/ml EGF for 6

and 12 days. Desmoplakin, a component of desmosomes (Cowin *et al.*, 1984), was immunohistochemically stained to identify cellular boundaries. As shown in Figure 4.3a and 4.3b, mononucleated cells dominated the EGF- cultures. There are few, if any, multinucleated syncytiotrophoblasts up to 12 days in the absence of EGF. In contrast, cultures in the presence of EGF developed into multinucleated giant cells with increasing frequency as the culture time increases from 6 days to 12 days (Figures 4.3c and 4.3d). The nuclei of such syncytialized cells are clustered in groups and separated by large expanses of cytoplasm. Mononucleated cells were still present in such cultures, but syncytialized cells dominated. Therefore, EGF could induce the differentiation of CTs into STs as early as 6 days, a process which is very slow in EGF- medium.

To clarify the expression pattern of Bcl-2 protein in cytotrophoblasts and syncytiotrophoblasts, purified first trimester and term CTs were cultured either in medium alone or in medium supplemented with 10 ng/ml EGF to induce syncytialization. As shown in Figures 4.4, there was no change in the amount of Bcl-2 protein in first trimester CTs and STs up to 12 days. The same was observed in term trophoblasts (Figure 4.5). Similar results were obtained in three different batches of each type of placenta. Therefore, Bcl-2 expression was the same in CTs and STs from first trimester or term placentas. This suggested, in addition to expression invariability with developmental stages of the placenta, Bcl-2 was also invariable with the differentiation states of the trophoblasts.

Bcl-2 staining in trophoblast cultures is patchy

Although the immunoblot analyses suggested that Bcl-2 protein levels were the same in cytotrophoblasts and syncytiotrophoblasts, the method measures the population as a whole. There remains the possibility that at the individual cell level Bcl-2 expression is variable. In order to study its expression pattern *in vitro*,

trophoblasts from first trimester and term placentas were cultured with or without 10 ng/ml EGF. The resulting CT or ST cultures were immunohistochemically stained with anti-Bcl-2 antibody at days 3, 6, 9, and 12. As shown in Figure 4.6, the staining pattern is similar between first trimester and term CTs and STs at each time point. The staining is cytoplasmic and perinuclear, consistent with the reported intracellular localization of Bcl-2 (Monaghan *et al.*, 1992; Nguyen *et al.*, 1993; Krajewski *et al.*, 1993; Lithgow *et al.*, 1994; Akao *et al.*, 1994). At day 3 (Figure 4.6a), although some cells stained more strongly for Bcl-2 than others, staining was distributed relatively evenly in the culture. With increasing incubation time, the cell monolayer changes as cells form tight junctions and syncytial units, strong Bcl-2 staining manifests as patches. The observation is most pronounced at day 12, where there are areas strongly stained for Bcl-2, while in other areas there is virtually no staining. These data suggest the population of cells is heterogeneous and consisting of high and low Bcl-2 expressing cells.

Bcl-2 expression of cytotrophoblasts appears homogeneous when assessed by FACS

In order to independently assess the distribution of Bcl-2 protein expression in cytotrophoblasts, freshly isolated and purified cytotrophoblasts from first trimester and term placentas were subjected to flow cytometric analysis (FACS). This method allows the measurement of the content of a particular protein in individual cells. Cells were immunofluorescence stained with anti-Bcl-2 antibody conjugated to FITC or isotype control antibody-FITC. Figure 4.7 contains typical profiles of Bcl-2 protein content in first trimester and term CTs. Specific staining is approximately one logarithm above the isotype control. Both profiles are similar and do not suggest any heterogeneity in Bcl-2 protein content since the profile distributions are relatively symmetrical. There is no evidence for a distinct

population of cells containing high levels of Bcl-2. There remains the possibility that the difference is too subtle for FACS to discern, and that immunohistochemical staining may be more suited for visual interpretation, particular after prolonged incubation of the cultures.

Section conclusion

From the data presented in this section, it can be concluded that Bcl-2 is expressed to the same level in total cultures of both first trimester and term cytotrophoblasts and syncytiotrophoblasts (Figures 4.1, 4.2, 4.4, and 4.5). However, there is evidence that heterogeneity exists in Bcl-2 content of individual cells (Figure 4.6).

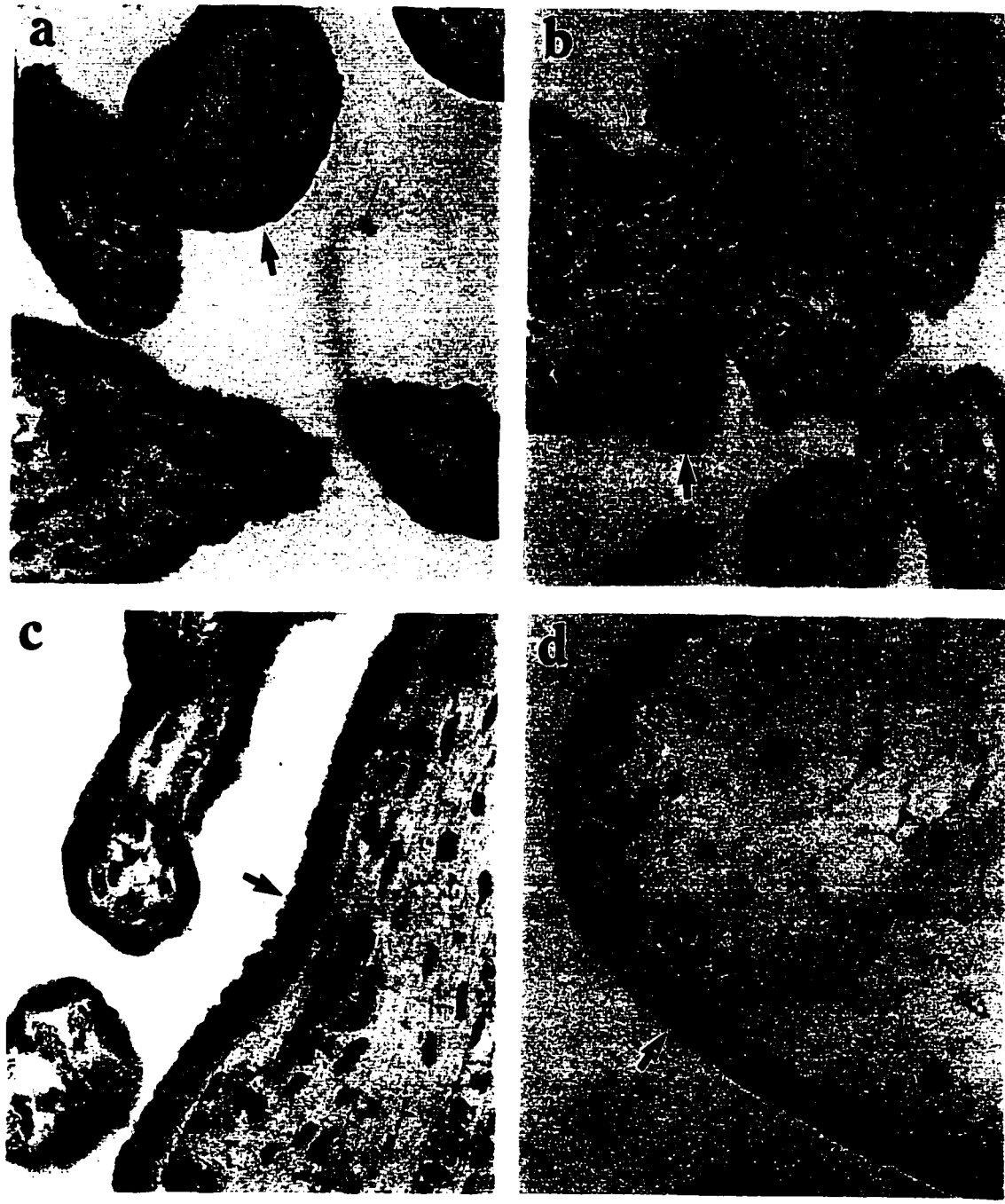

a
b
c
d

Figure 4.1 Tissue section staining of Bcl-2 protein in first trimester and term placental villi. Placental villi were isolated from term (panels a and b) and first trimester (c, d) placentas and immunohistochemically stained for the Bcl-2 protein (a, c) as described in Materials and Methods. b and d were stained with isotype control antibody. The arrows indicate the trophoblast layer. Similar results were obtained in six different placental sections.

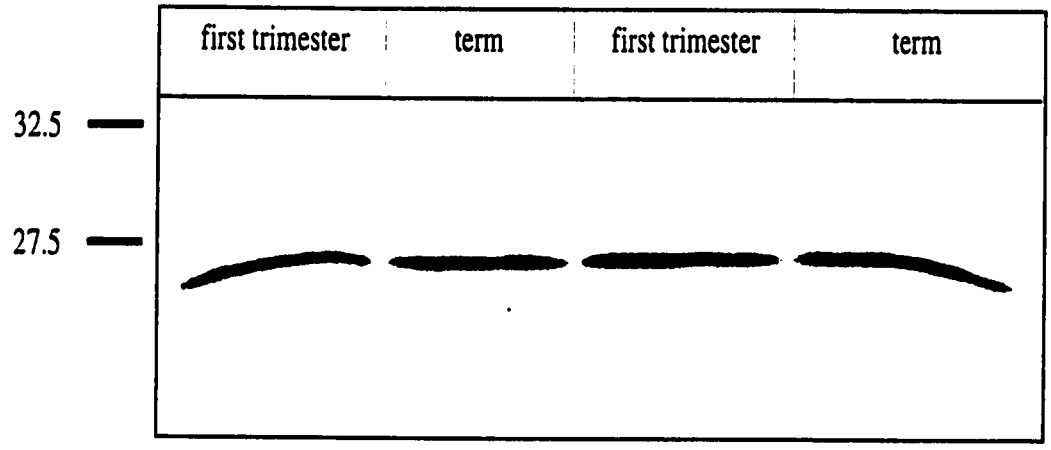


Figure 4.2 Immunoblot analysis of Bcl-2 protein expression in trophoblasts from first trimester and term placentas. Four different batches of freshly isolated and purified first trimester and term cytotrophoblasts were lysed in lysis buffer and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein were loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size markers in kDa are shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein. The data shown are representative of three separate experiments.

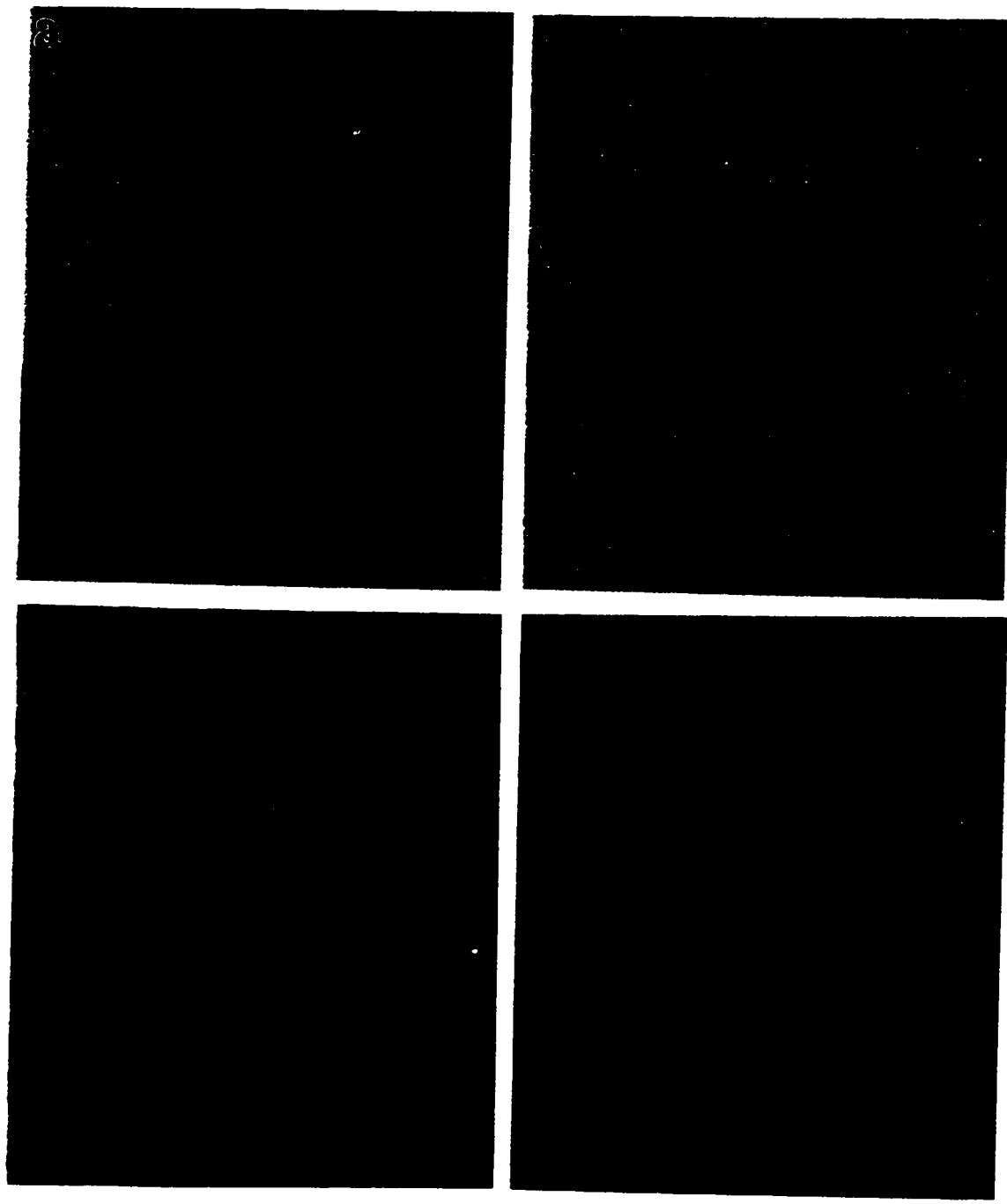


Figure 4.3 Immunohistochemical staining of desmoplakin protein in cultured term trophoblasts. Purified trophoblasts from term placentas were cultured in either medium (10% FCS/IMDM) alone (panels a and b) or medium supplemented with 10 ng/ml EGF (c, d). Cells were stained immunohistochemically on day 6 (a, c) and day 12 (b, d) of culture for desmoplakin protein or with an isotype control antibody (not shown) as described in Materials and Methods. Similar results were obtained in two separate experiments.

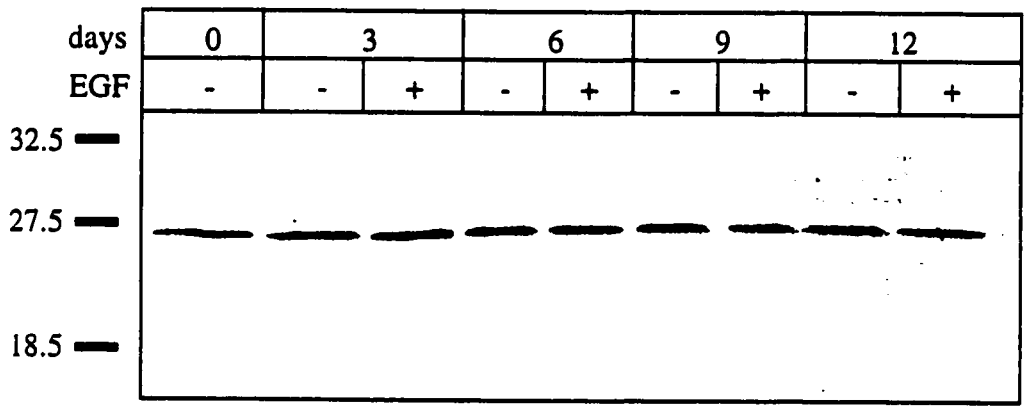


Figure 4.4 Immunoblot analysis of Bcl-2 protein expression in cultured first trimester cytotrophoblasts and syncytiotrophoblasts. Purified trophoblasts from first trimester placenta were cultured in medium (10% FCS/IMDM) alone (-) or in medium supplemented with 10 ng/ml EGF (+) to induce differentiation into syncytiotrophoblasts. Fresh medium was added every 3 days. Cells were lysed on the days shown and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein were loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size markers in kDa are shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein. The data shown are representative of three separate experiments.

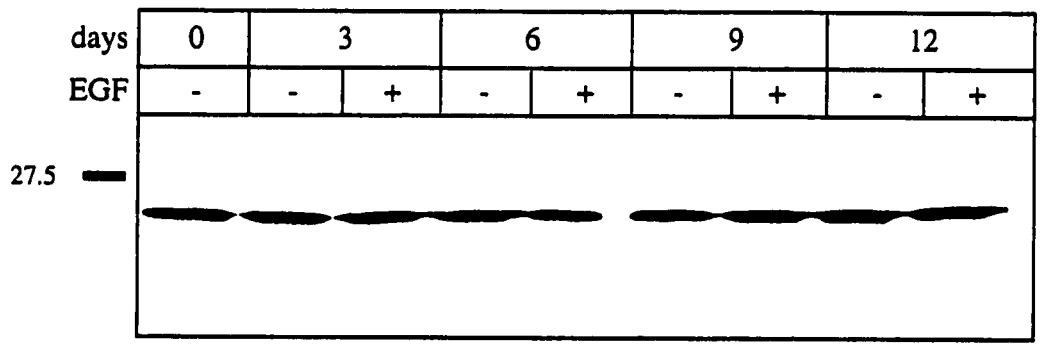
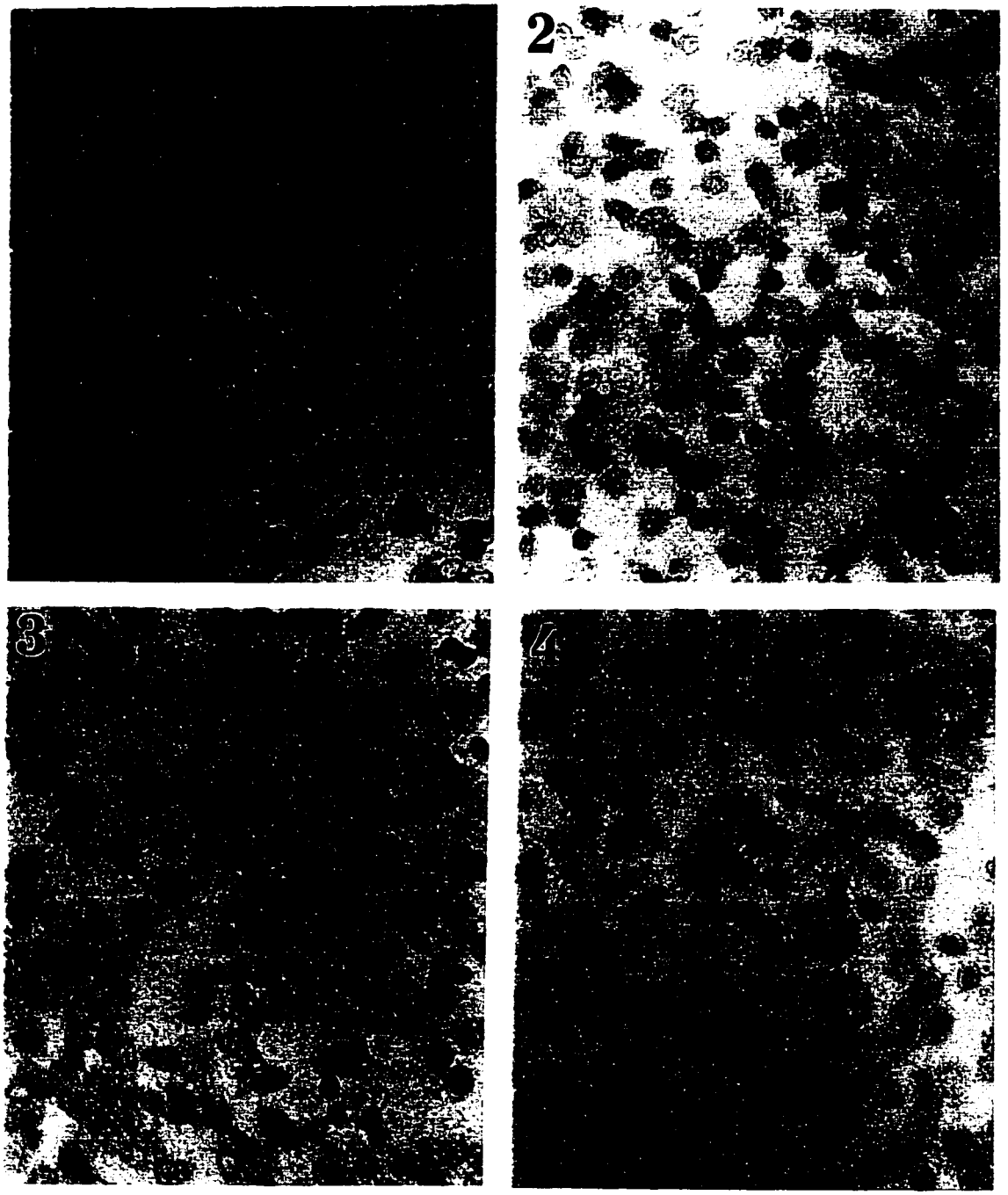


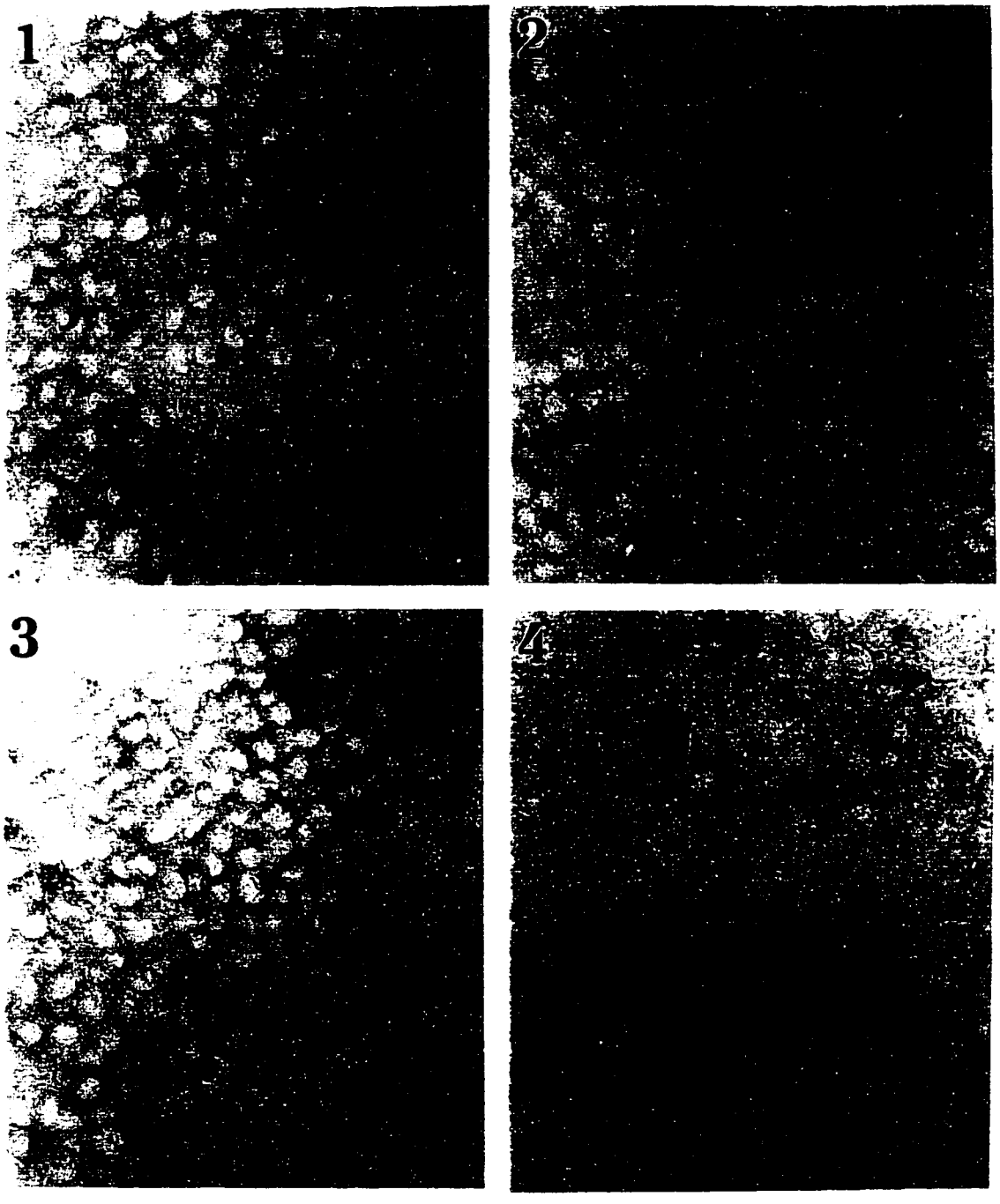
Figure 4.5 Immunoblot analysis of Bcl-2 protein expression in cultured term cytotrophoblasts and syncytiotrophoblasts. Purified trophoblasts from term placenta were cultured in medium (10% FCS/IMDM) alone (-) or in medium supplemented with 10 ng/ml EGF (+) to induce differentiation into syncytiotrophoblasts. Fresh medium was added every 3 days. Cells were lysed on the days shown and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein were loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size marker in kDa is shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein. The data shown are representative of three separate experiments.

a) Day 3



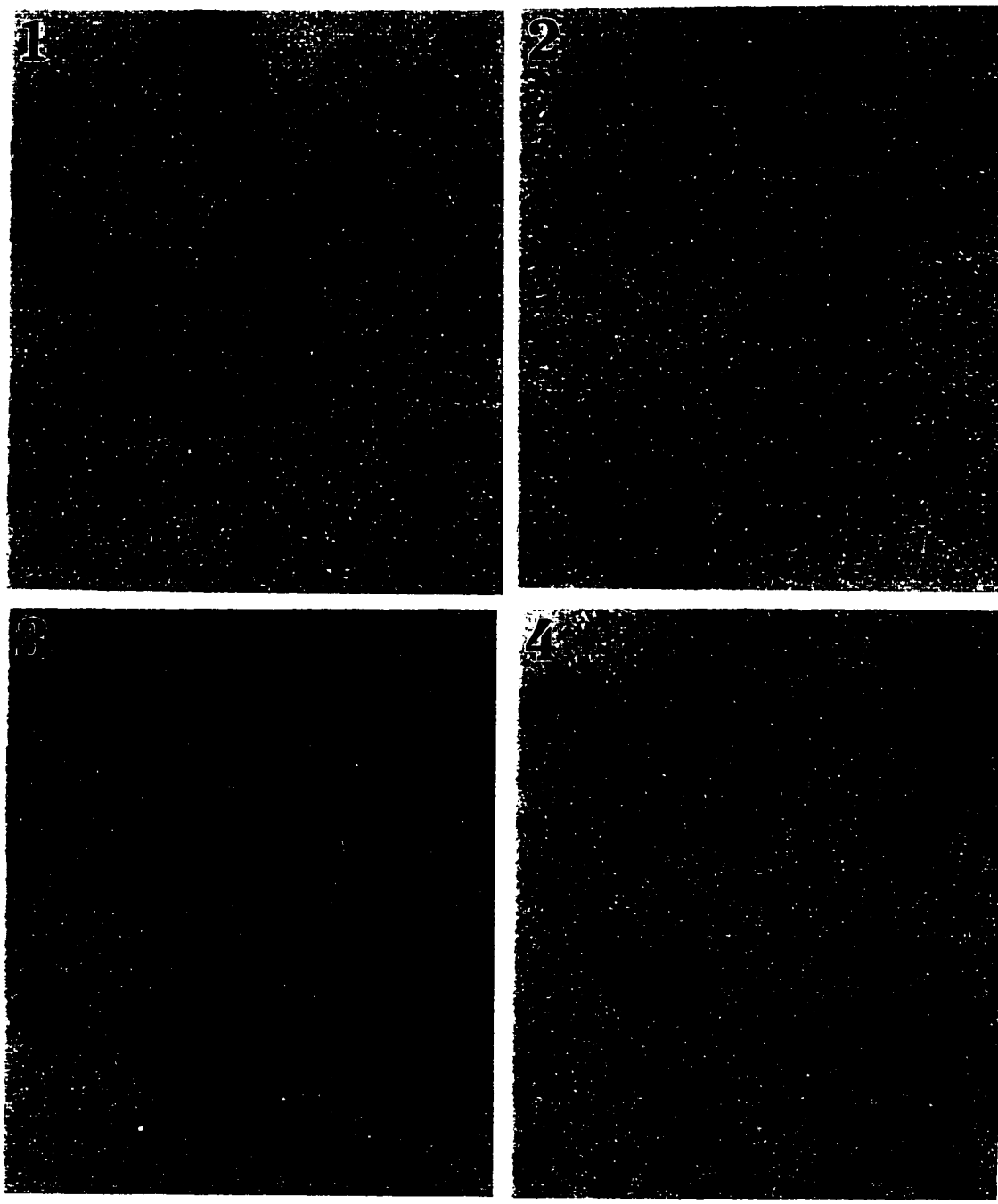
1. first trimester; - EGF
2. first trimester; + EGF
3. term; - EGF
4. term; + EGF

b) Day 6



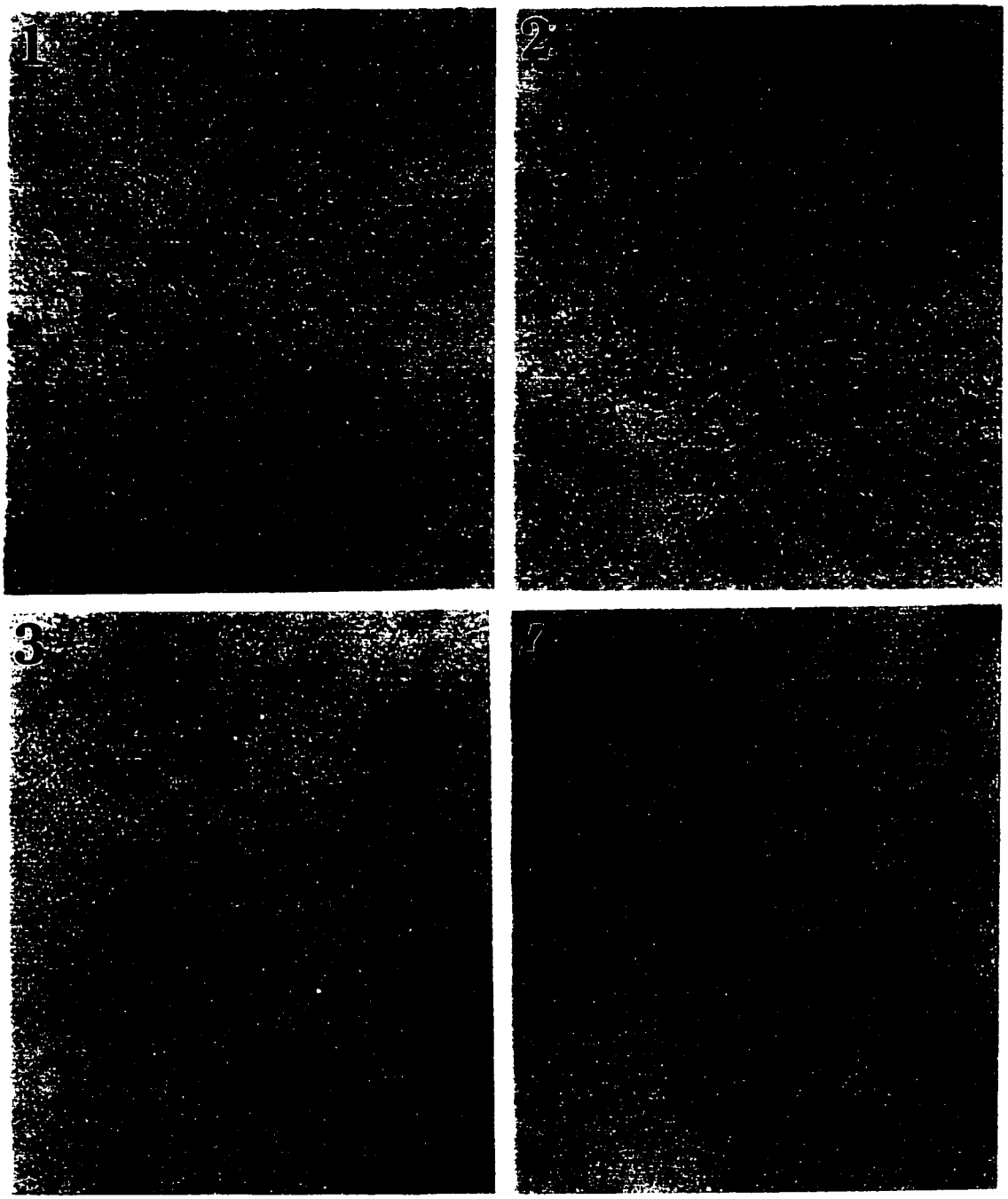
1. first trimester; - EGF
2. first trimester; + EGF
3. term; - EGF
4. term; + EGF

C) Day 9



1. first trimester; - EGF
2. first trimester; + EGF
3. term; - EGF
4. term; + EGF

d) Day 12



1. first trimester; - EGF
2. first trimester; + EGF
3. term; - EGF
4. term; + EGF

Figure 4.6 Immunohistochemical staining of Bcl-2 protein in cultured first trimester and term cytotrophoblasts and syncytiotrophoblast. Purified trophoblasts from first trimester (panels 1 and 2) and term placentas (3, 4) were cultured in either medium (10% FCS/IMDM) alone (1, 3) or in medium supplemented with 10 ng/ml EGF (2, 4). On days 3 (a), 6 (b), 9 (c), and 12 (d), cells were stained immunohistochemically for the Bcl-2 protein as described in Materials and Methods. Staining with isotype control antibody (not shown) indicates Bcl-2 staining is specific. Similar results were obtained in two separate experiments.

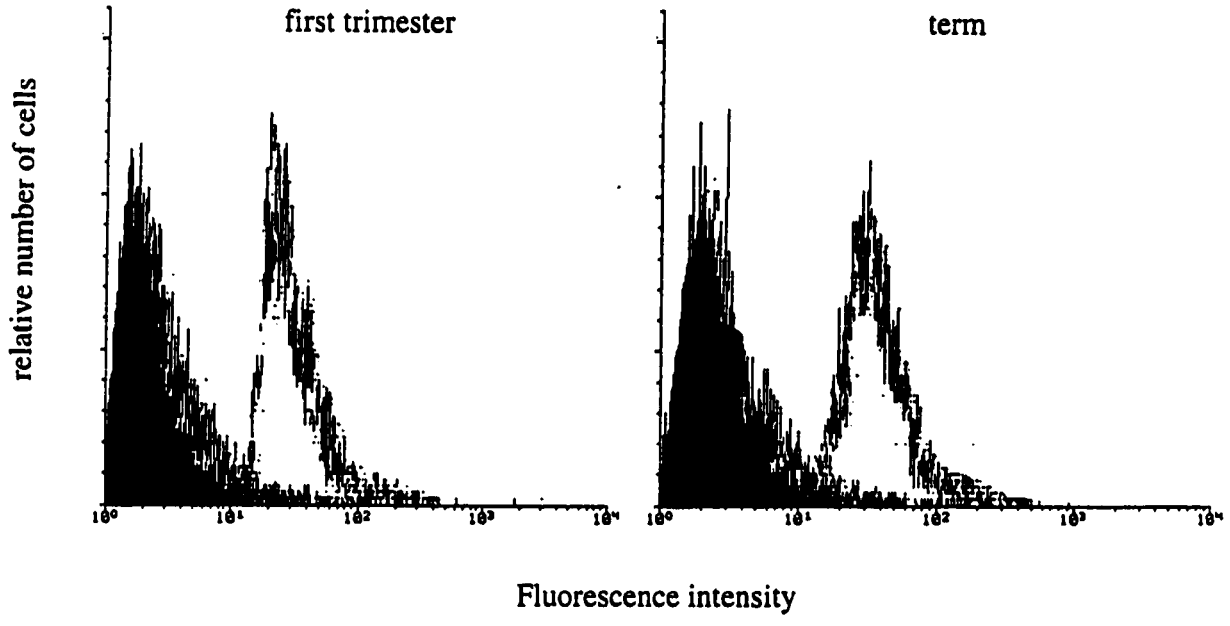


Figure 4.7 Flow cytometric analysis of Bcl-2 protein expression in trophoblasts from first trimester and term placentas. Freshly isolated and purified first trimester and term cytotrophoblasts were immunofluorescence-stained with either monoclonal mouse anti-human Bcl-2 antibody (Dako) conjugated to FITC (white area) or FITC-conjugated isotype control antibody (Dako; black area). The experiment was repeated using four different batches of trophoblasts with similar results.

4.2 THE EFFECT OF EXTERNAL AGENTS ON Bcl-2 EXPRESSION

The data presented in the previous section shows that the expression of Bcl-2 in trophoblasts is relatively constant regardless of differentiation and gestational state. Bcl-2 expression is constant *in vivo* and in total cultures *in vitro*, even when stimulated to differentiate with EGF. Therefore, under normal physiological conditions, the protein's expression is constitutive and stable. I next asked whether external stimuli such as cytokines and metabolic drugs known to affect trophoblast survival can alter Bcl-2 protein levels.

Inflammatory cytokines that induce apoptosis in cultured trophoblast do not alter Bcl-2 expression

Yui *et al.* (1994 b) have found that the inflammatory cytokine TNF-α causes apoptosis in trophoblasts. EGF, a cytokine advantageous to placental and trophoblast growth and well being, can suppress such cell death (Garcia-Lloret *et al.*, 1996). The mechanisms of TNF-α-induced apoptosis and EGF-mediated protection in trophoblasts are unknown at present. A strong candidate is the anti-apoptotic properties of Bcl-2. A central hypothesis in this thesis is that Bcl-2 protein level is down-regulated in trophoblasts during TNF-α-mediated apoptosis to allow the cell death program to proceed. Conversely, EGF up-regulates or maintains Bcl-2 protein levels to oppose the cell death program. Before proceeding to test this hypothesis, the ability of EGF, TNF-α and IFN-γ to regulate trophoblast apoptosis was reexamined. The results are presented in Figure 4.8. Term trophoblasts were cultured with EGF, TNF-α, IFN-γ, or their combinations for 24 hours and subjected to TUNEL for cell death analysis. This method measures DNA nicking, characteristic of apoptosis, by labeling free DNA ends with biotinylated UTP, a reaction carried out by terminal transferase. As shown in

Figure 4.8, TNF-α induces strong DNA nicking in 20% of cell, while co-culture with TNF-α and IFN-γ elevates apoptosis to 50%. IFN-γ alone does not cause significantly more cell death above control levels. When EGF is also present, TNF-α/IFN-γ-induced apoptosis is reduced to 10%. Throughout this work, TNF-α is always accompanied by IFN-γ for induction of maximum cell death.

After establishing the reproducibility of TNF-α/IFN-γ-induced apoptosis, the hypothesis of cytokine-regulated Bcl-2 expression was tested. First trimester and term trophoblasts were cultured with EGF, TNF-α/IFN-γ, or combination of them for 1, 2, and 3 days. Immunoblots were performed on cell lysates with anti-Bcl-2 antibody to determine Bcl-2 protein levels. As shown in Figures 4.9 and 4.10, respectively, first trimester and term trophoblasts show no change in Bcl-2 content under all conditions tested and at all time points. Therefore, Bcl-2 expression is invariable in the face of cytokine stimulation. TNF-α and EGF do not appear to exert their effects on trophoblasts by modulating Bcl-2 protein level. There remains the possibility that cells prone to apoptosis have an initially low Bcl-2 content, raising the issue of trophoblast heterogeneity examined earlier. Alternatively, the cytokines might cause post-translational modifications of the Bcl-2 protein and affecting its function. For instance, Bcl-2 has been demonstrated to be phosphorylated, but the significance of this is unclear (Haldar *et al.*, 1995; Ito *et al.*, 1997).

Cycloheximide has no effect on Bcl-2 expression

Yui (1994) found that trophoblasts are extremely sensitive to cycloheximide (CHX), an inhibitor of protein synthesis. At a sublethal dose of 300 ng/ml, it enhances TNF-α/IFN-γ-induced cell death, indicating the cell death machinery is already present in trophoblasts but normally suppressed by short-lived proteins (Yui, 1994). To test whether CHX affects Bcl-2 expression, term trophoblasts

were cultured in 300 ng/ml CHX or TNF-α/IFN-γ plus CHX for 48 hours before immunoblotting for Bcl-2 (Figure 4.11). The total protein from each harvest was approximately equal (around 100 μg; data not shown). In neither conditions are there changes in Bcl-2 content. Therefore, CHX treatment potentiate TNF-α/IFN-γ-induced apoptosis without affecting Bcl-2 protein levels.

GM-CSF and dexamethasone have no effect on Bcl-2 expression

In addition to EGF, GM-CSF (granulocyte-macrophage colony stimulating factor) is also associated with beneficial effects on pregnancy and placental well being (Chaouat *et al.*, 1990; Loke *et al.*, 1992; Garcia-Lloret *et al.*, 1994), but it does not significantly inhibit TNF-α/IFN-γ-mediated apoptosis in trophoblasts (Yui, 1994; Garcia-Lloret *et al.*, 1996). On the other hand, the glucocorticoid dexamethasone can suppress TNF-α/IFN-γ-induced trophoblast apoptosis as well as EGF. Could these agents confers protection to trophoblasts by up-regulating Bcl-2? To answer this question, term trophoblasts were cultured in two different concentrations of each of GM-CSF and dexamethasone. Cells were lysed after 36 hours and immunoblotted for Bcl-2. As shown in Figure 4.12, Bcl-2 protein levels were unchanged in all conditions. Therefore these agents have no effect on Bcl-2 expression and they mediate cellular well-being via other pathways.

Section conclusion

TNF-α/IFN-γ-induced apoptosis is not accompanied by Bcl-2 protein down-regulation, nor does EGF confer protection by up-regulating the protein (Figures 4.9 and 4.10). Bcl-2 expression is constant and stable after treatments with cycloheximide, GM-CSF, and glucocorticoid (Figures 4.11 and 4.12).

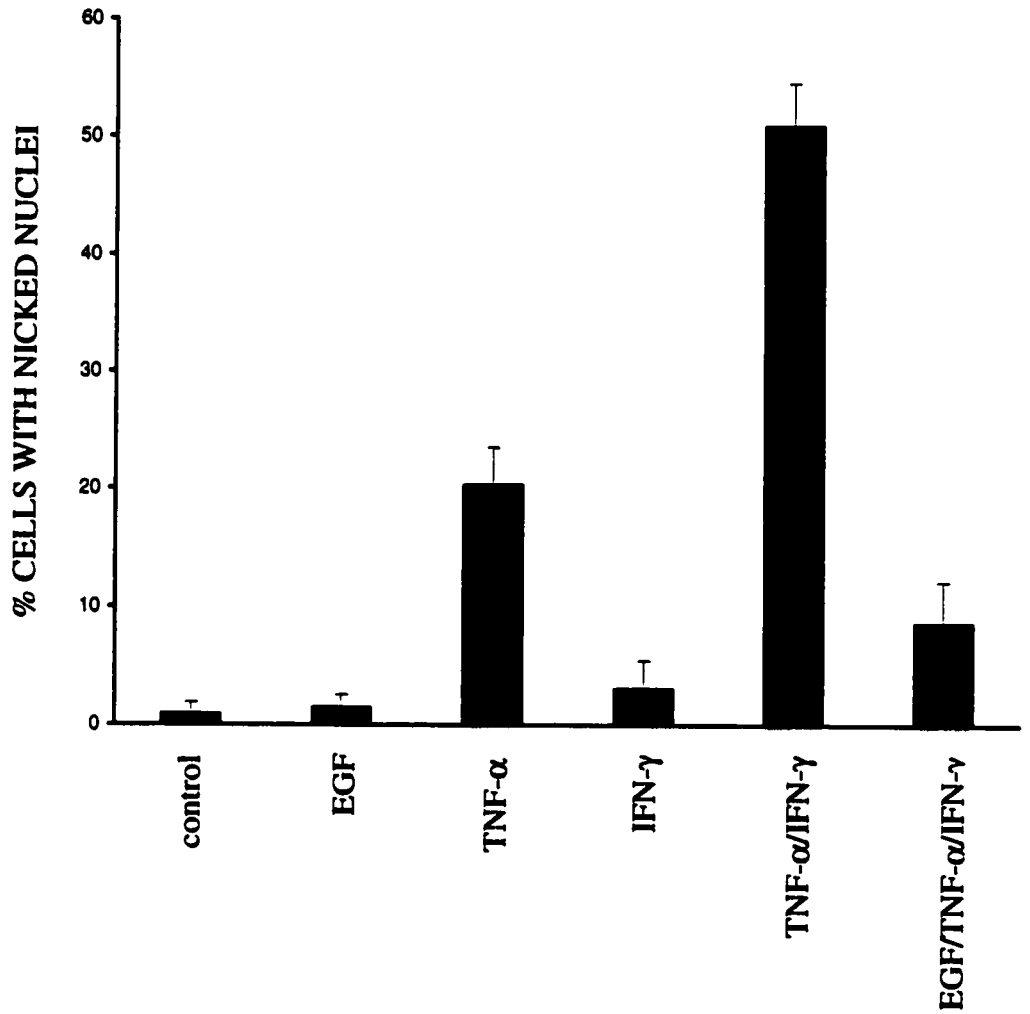


Figure 4.8 TUNEL analysis of trophoblast apoptosis in cultures with EGF, TNF-α, or IFN-γ. Purified trophoblasts from term placenta were cultured in either medium (10% FCS/IMDM) alone (control), medium supplemented with 10 ng/ml EGF, with 10 ng/ml TNF-α, with 100 U/ml IFN-γ, with both TNF-α plus IFN-γ, or with EGF and TNF-α plus IFN-γ for 24 hours before performing TUNEL as described in Materials and Methods. A total of 1500-2000 cells from 10 different fields in two separate experiments were counted. Bars represent the mean percentage of cells with nicked DNA ± S.D.

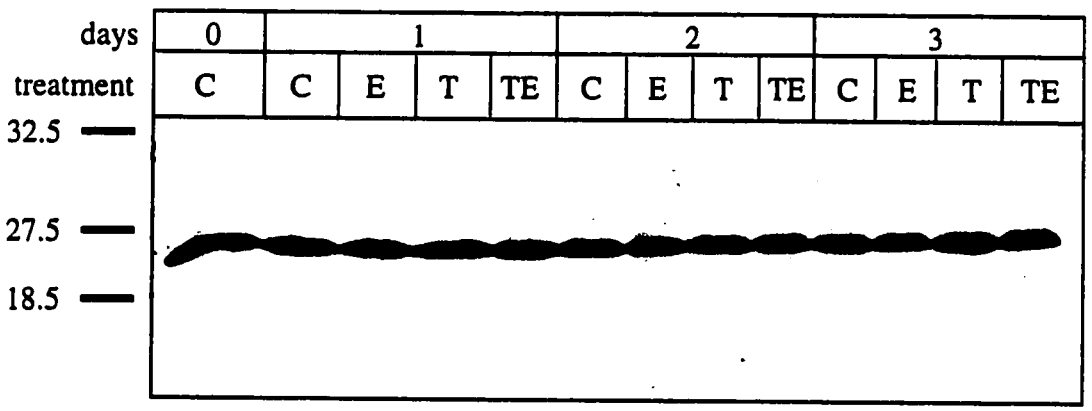


Figure 4.9 Immunoblot analysis of Bcl-2 protein expression in first trimester trophoblasts cultured in EGF, TNF-α/IFN-γ, or both. Purified trophoblasts from first trimester placenta were cultured in medium (10% FCS/IMDM) alone (control; C), medium supplemented with 10 ng/ml EGF (E), with 10 ng/ml TNF-α plus 100 U/ml IFN-γ (T), or with both EGF and TNF-α plus IFN-γ (TE). Cells were lysed on the days shown and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein were loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size markers in kDa are shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein. The data shown are representative of three separate experiments.

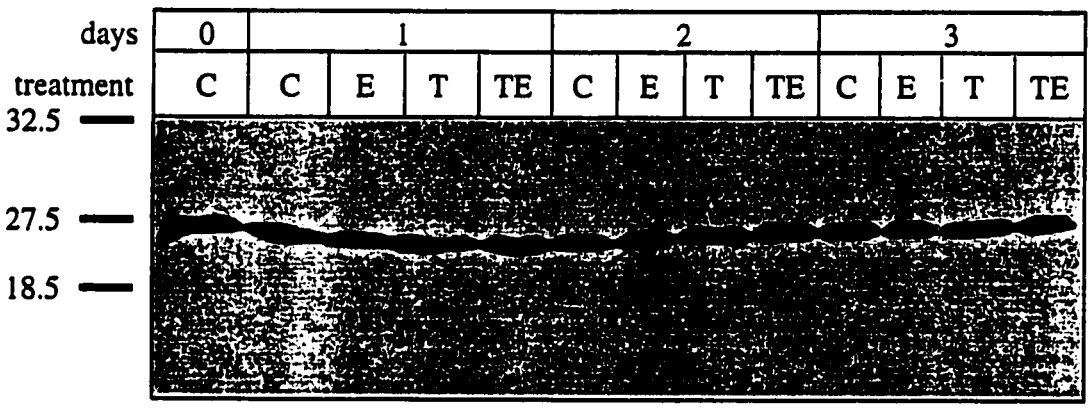


Figure 4.10 Immunoblot analysis of Bcl-2 protein expression in term trophoblasts cultured in EGF, TNF-α/IFN-γ, or both. Purified trophoblasts from term placenta were cultured in medium (10% FCS/IMDM) alone (control; C), medium supplemented with 10 ng/ml EGF (E), with 10 ng/ml TNF-α plus 100 U/ml IFN-γ (T), or with both EGF and TNF-α plus IFN-γ (TE). Cells were lysed on the days shown and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein were loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size markers in kDa are shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein. The data shown are representative of three separate experiments.

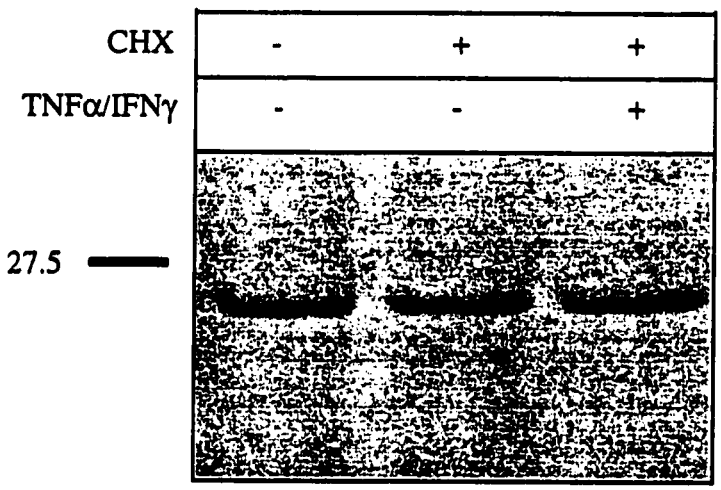


Figure 4.11 Immunoblot analysis of Bcl-2 protein expression in term trophoblasts cultured in cycloheximide or cycloheximide plus TNF-α/IFN-γ. Purified trophoblasts from term placenta were cultured in medium (10% FCS/IMDM) alone, medium supplemented with 300 ng/ml cycloheximide, or cycloheximide with 10 ng/ml TNF-α plus 100 U/ml IFN-γ for 48 hours. Cells were lysed and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein were loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size marker in kDa is shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein. The data shown are representative of three separate experiments.

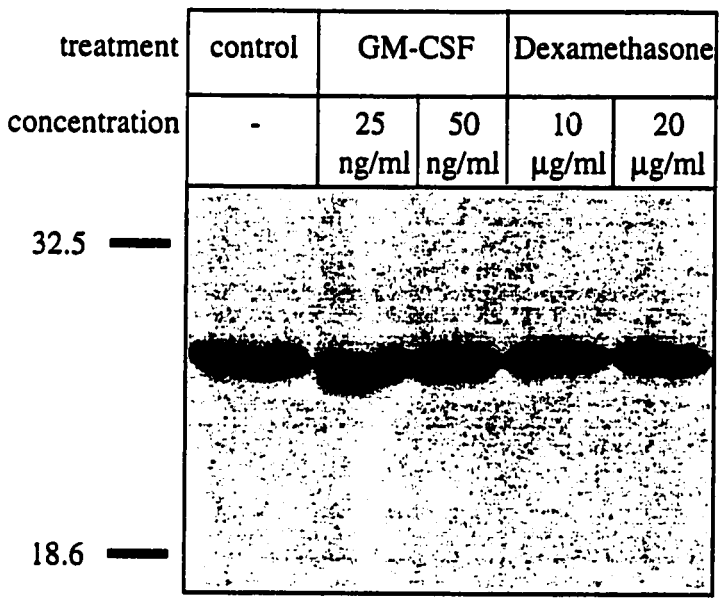


Figure 4.12 Immunoblot analysis of Bcl-2 protein expression in term trophoblasts cultured in GM-CSF or dexamethasone. Purified trophoblasts from term placenta were cultured in medium (10% FCS/IMDM) alone, medium supplemented with 25 or 50 ng/ml GM-CSF, or with 10 or 20 µg/ml dexamethasone for 36 hours. Cells were lysed and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein were loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size markers in kDa are shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein. The data shown are representative of three separate experiments.

4.3 PREDISPOSITION OF TROPHOBLASTS TO APOPTOSIS

Results from the previous sections show that Bcl-2 protein levels are remarkably stable in a number of different stimulation and maturation states. Trophoblast apoptosis or protection from apoptosis is not accompanied by changes in Bcl-2 expression as measured by immunoblotting. Nonetheless, there is evidence that, despite being constitutively expressed at the pooled population level, there is heterogeneity in Bcl-2 content among individual cells. This heterogeneity could mean that cells expressing low levels of Bcl-2 are predisposed to killing by TNF-α/IFN-γ. In this section, this hypothesis is examined.

Measurement of apoptosis using FACS

Flow cytometric analysis is a powerful tool for the study of individual cells in a population. One-, two-, or three-color fluorescence staining allows the correlation of multiple parameters. Cells undergoing apoptosis can be detected using FACS by staining DNA with propidium iodide (PI). PI fluorescence is site activated, meaning it will be fluorescence-activatable only when bound to its target, DNA. Therefore, this method allows the direct measurement of DNA content in a cell. When a cell undergoes apoptosis, its DNA is nicked and fragments are extruded from the cell (Wyllie *et al.*, 1980; Savill *et al.*, 1993). Any cell that contains less than 2n (diploid) DNA content can be considered to be undergoing apoptosis. The feasibility of this method in measuring trophoblast apoptosis was first examined. Purified term trophoblasts were cultured for 24 hours either in medium alone, or medium supplemented with EGF, TNF-α/IFN-γ, or EGF plus TNF-α/IFN-γ. Cells were then stained with PI and FACS profiles of DNA content obtained (Figure 4.13). Small cellular debris such as membrane fragments have been gated out of the FACS analysis. Healthy cells with 2n DNA

gave fluorescence intensity of 200-250 (arbitrary units assigned by FACS). Cells are defined as apoptotic if they contained less DNA than 95% of the healthy cells, and cells with values in multiples of 200-250 have probably been cross-linked together during fixing or are syncytial units with more than two nuclei, giving 4n or more DNA content. As shown in Figure 4.13, by definition 5% of cells are apoptotic (contain < 2n) in control and EGF treated cells, and 7% of cells are dead in the presence of EGF plus TNF-α/IFN-γ. In TNF-α/IFN-γ treated cells, there is 12% apoptosis, more than double that of control. This value is substantially less than the value obtained by TUNEL analysis (50%; Figure 4.8). It is likely due to the nature of the measurement by the two methods. The criteria for apoptosis is DNA cleavage for both methods. However, TUNEL measures DNA nicking independent of the degree that would lead to fragment loss. TUNEL is more sensitive than PI-staining because a cell that has cleaved its DNA, but has not yet disposed of it, is registered as non-apoptotic. In PI-staining, not until a cell has less than 2n DNA would it be considered positive for cell death. A second source of insensitivity could be that apoptotic cells are more fragile than non-apoptotic cells, and thus could be preferentially lost during harvesting of cells for analysis. Alternatively, the insensitivity could also be due to fusion of dead cells with each other or with live cells, giving values in between multiples of 200 fluorescence intensity. Nevertheless, PI-staining can be used for the measurement of apoptosis in trophoblasts, since there is a large enough difference between control and TNF-α/IFN-γ-treated cells.

Low Bcl-2 content predisposes trophoblasts to apoptosis

In order to determine if there is a correlation between Bcl-2 protein content and susceptibility to TNF-α/IFN-γ-induced apoptosis in the trophoblasts, double fluorescence staining of Bcl-2 and DNA was developed (Figure 4.14). There are

no distinct subpopulations of high and low Bcl-2 expressing cells measurable by the analyses in Sections 4.1 and 4.2. Thus, the FACS profile of Bcl-2 protein content was divided into approximate halves to denote the two subpopulations. Purified term trophoblasts were cultured for 24 hours either in medium alone, or medium supplemented with EGF, TNF-α/IFN-γ, or EGF plus TNF-α/IFN-γ. Cells were then stained with FITC-conjugated anti-Bcl-2 antibody followed by PI, and the FACS profiles of Bcl-2 and DNA contents obtained. Figure 4.14a is a typical FACS profile of PI staining on control cells and shows the gate (less than a fluorescence intensity of 200) that defines apoptotic cells. A cell was defined as apoptotic if it contained less DNA than 95% of the cells with 2n DNA content (healthy). Figure 4.14b is a typical FACS profile of Bcl-2 content for control cells. The black curve is specific Bcl-2 staining and the white curve is isotype control antibody staining. To define high and low Bcl-2 expressing cells, a gate is set at FITC fluorescence intensity of 5, which is the peak intensity channel, and where there is minimum overlap with the isotype curve. Approximately half of the population falls to either side of it. 46% of trophoblasts have higher than a fluorescence intensity of 5, and are defined as high Bcl-2 expressing cells. Each of high and low expressing population occupies approximately five units of staining intensity. Figures 4.14c-f are Bcl-2/DNA content scatter plots for the four culture conditions tested. On the Y-axis is PI fluorescence intensity (DNA content) and on the X-axis is FITC fluorescence intensity (Bcl-2 content). Each dot on the plot represents an individual cell with its corresponding Bcl-2 and DNA contents. The lines represent gates defined in Figures 4.14a and b. The upper-left quadrant (UL) represents cells that are low in Bcl-2 and non-apoptotic; upper-right quadrant (UR) means high in Bcl-2 and non-apoptotic; lower-left quadrant (LL) is low in Bcl-2 and apoptotic; and lower-right quadrant (LR) is high in Bcl-2 and apoptotic. The percentages of cells in each quadrant are indicated.

To determine if predisposition to cell death exists in low and high Bcl-2 expressing trophoblasts, the percentages of apoptotic cells in each subpopulation are calculated. For % apoptosis in low Bcl-2 expressors, the formula LL/(UL+LL) x 100 is used; for % apoptosis in high Bcl-2 expressors, the formula LR/(UR+LR) x 100 is used. The results are summarized in Table 4.1. Cell death remains between 4% to 6% for high Bcl-2 expressors in all conditions tested, indicating cells with high Bcl-2 content are relatively resistant to TNF-α/IFN-γ-induced apoptosis. In contrast, in low Bcl-2 expressors, apoptosis increases from 6% for the control to 21% when TNF-α/IFN-γ are present. Comparing high and low Bcl-2 expressors, TNF-α/IFN-γ brought about 6% and 21% cell death respectively. This means trophoblasts with low Bcl-2 content are more prone to TNF-α/IFN-γ-mediated apoptosis. It should be noted that during apoptosis, in addition to DNA, cellular proteins are also cleaved as an early step in deposing of a dead cell. Thus it is possible that the low Bcl-2 content is a consequence of cell death, instead of the cause of predisposition to apoptosis. However, as shown in Figures 4.9 and 4.10, populational Bcl-2 protein content is unchanged in TNF-α/IFN-γ treated cells. Therefore, the predisposition to cell death observed is a consequence of low Bcl-2 expression instead of its cause. In co-culture of EGF and TNF-α/IFN-γ, cell death drops from 21% to 13% for low expressors, while cell death for high expressors remains relatively constant. Bcl-2 content is unchanged in response to EGF (Figures 4.9 and 4.10). This implies that EGF protects trophoblasts by either enhancing processes downstream of Bcl-2 or affecting a separate pathway parallel to Bcl-2. This phenomenon was observed in trophoblasts from three different placentas and is summarized in Table 4.2. For cells under control conditions, low Bcl-2 expressing cells are 1.25 ± 0.25 times more likely to die than high Bcl-2 expressing cells. In contrast, when TNF-α/IFN-γ is present, low expressors are 3.7 ± 0.5 times more likely to commit apoptosis. Thus there exists functional

heterogeneity in Bcl-2 expression among trophoblasts. TNF-α increases the probability that cells with less Bcl-2 protein will undergo apoptosis. Whether this heterogeneity has any functional significance in the placenta is unclear. It is also unknown if this heterogeneity is extended into the syncytium. However, the relationship implies certain areas on the placenta that are more vulnerable to destruction by TNF-α/IFN-γ than others.

Section conclusion

Subtle heterogeneity exists in Bcl-2 protein expression in trophoblasts. Low Bcl-2 expressing cells are predisposed to apoptosis induced by TNF-α/IFN-γ.

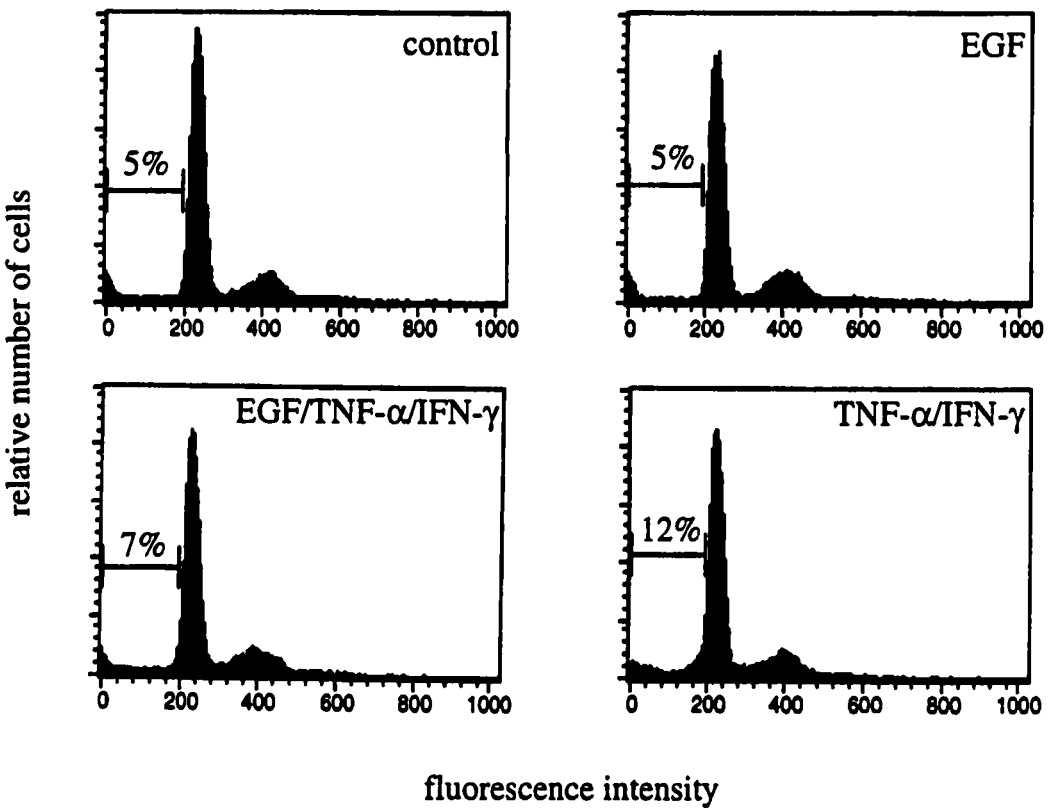


Figure 4.13 Flow cytometric analysis of DNA content in term trophoblasts cultured in EGF, TNF-α/IFN-γ, or both. Purified trophoblasts from term placenta were cultured in medium (10% FCS/IMDM) alone (control), medium supplemented with 10 ng/ml EGF, with 10 ng/ml TNF-α plus 100 U/ml IFN-γ, or with both EGF and TNF-α plus IFN-γ for 24 hours. Cells were fixed in 1% paraformaldehyde and stained with propidium iodide as described in Materials and Methods. The bars indicate the percentage of cells containing less than 2n DNA content. The experiment was repeated three times with similar results.

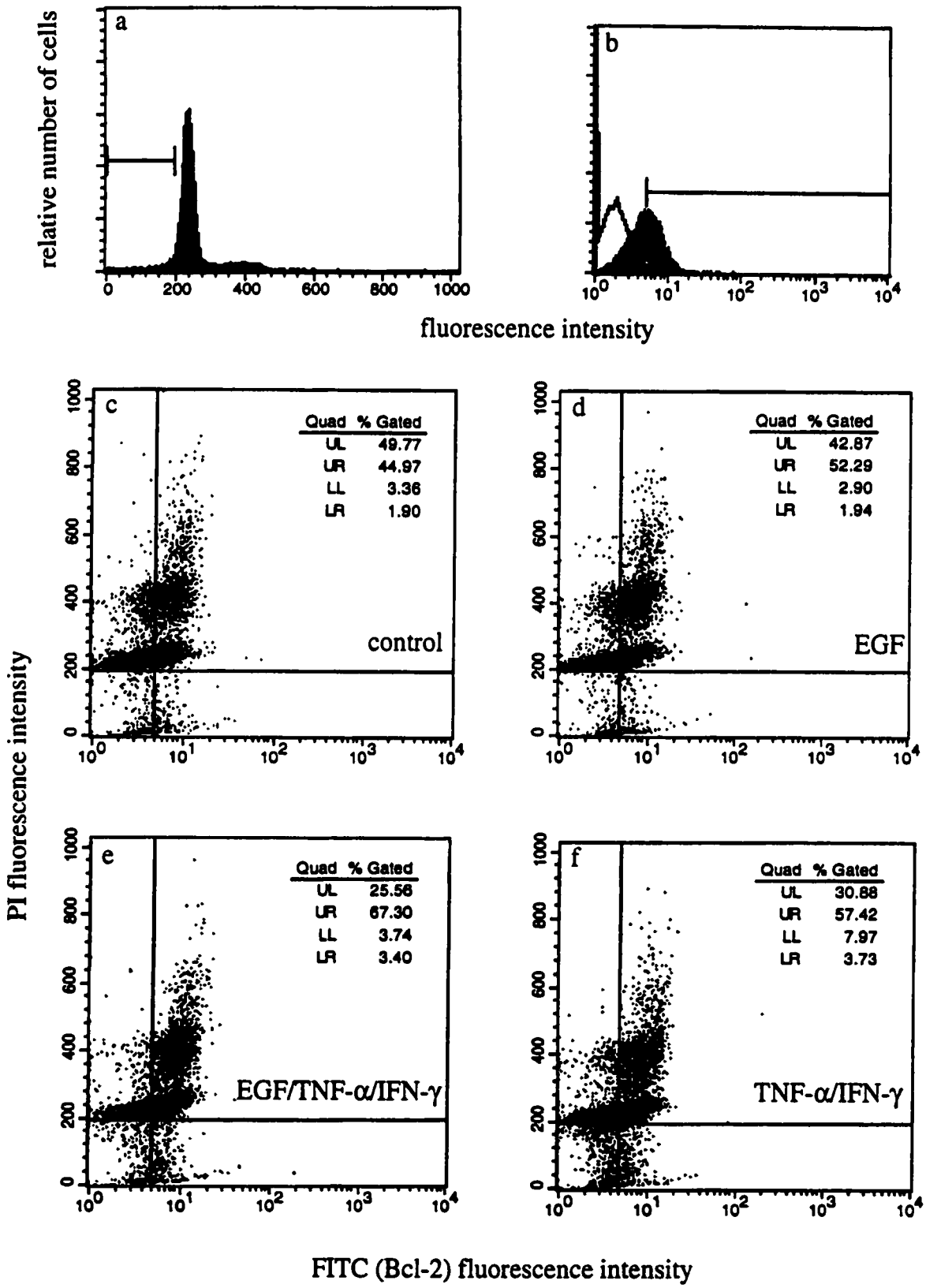

a
b
relative number of cells
fluorescence intensity
c
Quad % Gated
UL 49.77
UR 44.97
LL 3.36
LR 1.90
control
d
Quad % Gated
UL 42.87
UR 52.29
LL 2.90
LR 1.94
EGF
e
Quad % Gated
UL 25.56
UR 67.30
LL 3.74
LR 3.40
EGF/TNF-α/IFN-γ
f
Quad % Gated
UL 30.88
UR 57.42
LL 7.97
LR 3.73
TNF-α/IFN-γ
PI fluorescence intensity
FITC (Bcl-2) fluorescence intensity

Figure 4.14 Flow cytometric analysis of Bcl-2/DNA double stained term trophoblasts cultured in EGF, TNF-α/IFN-γ, or both. Purified trophoblasts from term placenta were cultured in either medium (10% FCS/IMDM) alone (control), medium supplemented with 10 ng/ml EGF, with 10 ng/ml TNF-α plus 100 U/ml IFN-γ, or with both EGF and TNF-α plus IFN-γ for 24 hours. Cells were doubly stained with monoclonal mouse anti-human Bcl-2 antibody (Dako) conjugated to FITC and propidium iodide (PI) as described in Materials and Methods. a) PI FACS profile of control cells; the bar indicates the region of cells containing less than 2n DNA content (apoptotic cell), the same region is used in panels c-f. b) FACS profile of control cells stained with anti-Bcl-2-FITC antibody (black area) or isotype control antibody-FITC (white area); the bar indicates the region of cells defined as High Bcl-2 Expressing Cells (46% of total population) in panels c-f. c-f) Bcl-2/DNA doubly stained FACS profile of cells cultured in medium alone (c), medium with EGF (d), EGF/TNF-α/IFN-γ (e), and TNF-α/IFN-γ (f). PI fluorescence intensity (DNA content) is displayed on the Y-axis and FITC fluorescence intensity (Bcl-2 protein content) is displayed on the X-axis. Percentages of cells in the upper-left (UL; low Bcl-2 and non-apoptotic), upper-right (UR; high Bcl-2 and non-apoptotic), lower-left (LL; low Bcl-2 and apoptotic), and lower-right (LR; high Bcl-2 and apoptotic) quadrants of each panel are shown. See Table 4.1 for analysis. The results shown are representative of three separate experiments.

Table 4.1 Comparison of cell death in high and low Bcl-2 expressing trophoblasts

% cell death	low Bcl-2 expressing cells	high Bcl-2 expressing cells
control	6%	4%
EGF	6%	4%
TNF-α/IFN-γ/EGF	13%	5%
TNF-α/IFN-γ	21%	6%

% cell death in low and high Bcl-2 expressing cells as defined in Figure 4.14 are calculated by the following formulas using values from Figure 4.14:

$$\%\ \text{cell death (low Bcl-2)} = \frac{LL}{UL+LL} \times 100$$

$$\%\ \text{cell death (high Bcl-2)} = \frac{LR}{UR+LR} \times 100$$

Table 4.2 Comparison of ratio of cell death in high and low Bcl-2 expressing trophoblasts

experiment	low Bcl-2 expressors	high Bcl-2 expressors	low/high ratio
1. Control	6%	4%	1.5
TNF-α/IFN-γ	21%	6%	3.5
2. Control	3%	3%	1
TNF-α/IFN-γ	25%	6%	4.2
3. Control	5%	4%	1.25
TNF-α/IFN-γ	30%	9%	3.3

mean ratio % cell death (low Bcl-2 expressors/high Bcl-2 expressors) ± S.D.:

Control: 1.25 ± 0.25

TNF-α/IFN-γ: 3.7 ± 0.5

- % cell death values from 3 separate experiments

4.4 TRANSFECTION OF TROPHOBLASTS

The observation that low Bcl-2 expression predisposes trophoblasts to TNF-α/IFN-γ-induced apoptosis suggests that increasing Bcl-2 levels will prevent apoptosis and *vice versa*. However as evident in Sections 4.1 and 4.2, Bcl-2 levels were remarkably stable and all external stimuli tested did not alter its expression. Moreover, since Bcl-2 is an intracellular protein and staining of it requires the fixing of cells, FACS cannot be used to sort trophoblast populations into low and high Bcl-2 expressors. The remaining option is to utilize transfection and overexpression methods to manipulate protein levels. Antisense DNA to Bcl-2 transcripts can potentially block the production of the protein, and full length sense Bcl-2 cDNA can increase Bcl-2 content of cells. Transfected trophoblast populations can be tested separately for vulnerability to TNF-α/IFN-γ-mediated apoptosis. This section documents the attempts at transfection.

Trophoblast can be transfected with β-galactosidase expression plasmids

The feasibility of transfection of primary trophoblast using calcium phosphate-DNA co-precipitation has been documented previously (Kilani *et al.*, 1997), by transfecting HIV provirus into trophoblasts with an efficiency of 5-15%. In order to verify the efficacy of the transfection method and estimate its efficiency, the β-galactosidase expression plasmid pcDNA3/Zeo/nlacZ (a kind gift from Dr. Lung-Ji Chang; Figure 3.2) was transfected into term trophoblasts. On day 3 post-transfection, the cells were stained with X-gal, a color-producing substrate of β-galactosidase. As shown in Figure 4.15, trophoblasts transfected with the β-galactosidase expression plasmid are stained blue, indicating calcium phosphate-DNA co-precipitation is a suitable method for transfecting and overexpressing exogenous proteins in trophoblasts. There is no staining in control

cells that are mock transfected (no plasmid DNA). The transfection efficiency was estimated at less than 10% in two experiments.

Overexpression of Bcl-2 in BeWo cells

Two Bcl-2 expression plasmids pR509-8-18 (antisense Bcl-2) and pR509-8-45 (sense Bcl-2) are used for transfection studies (Figure 3.2). They are kind gifts from Dr. John C. Reed. In order to test the functionality of the two plasmids, they were transfected into BeWo cells, a trophoblast cancer (choriocarcinoma) cell line. These cells express very low endogenous Bcl-2 (Figure 4.16) so they serve as good quality controls for the plasmids. Confluent BeWo cultures were transfected with antisense or sense Bcl-2, harvested 1, 2, 3, and 4 days post-transfection, and immunoblotted for Bcl-2. Control cells were mock transfected. As shown in Figure 4.16, by day 4, sense transfected BeWo cells overexpress Bcl-2. Therefore, the sense Bcl-2 plasmid is functional. Antisense transfected cells do not show an appreciable drop in Bcl-2 levels.

Trophoblast does not overexpress Bcl-2

Five separate attempts were made at transfecting and overexpressing Bcl-2 in trophoblasts. Term trophoblasts were transfected with either antisense or sense Bcl-2 expression plasmids and immunoblotted for Bcl-2 on day 5. As shown in Figure 4.17, there is no noticeable difference between control and transfected cells in Bcl-2 protein levels. However, since trophoblasts already contain high Bcl-2 at the population level, a 10% transfection and overexpression efficiency may not be discernible by immunoblot analysis. Therefore, transfected cells were immunofluorescence-stained for Bcl-2 and subjected to FACS analysis as described in Section 4.1. As shown in Figure 4.18a, there is no difference between the Bcl-2 FACS profiles of control and sense transfected cells with no evidence for

a small population of overexpressing cells at a higher fluorescence intensity. Comparison between the profiles of sense and antisense transfected cells also yields no noticeable difference. Therefore, primary trophoblast Bcl-2 protein level cannot be manipulated using these transfection methods.

Section conclusion

Trophoblasts can be transfected using calcium phosphate-DNA co-precipitation (Figure 4.15), but attempts at overexpressing Bcl-2 have been futile (Figures 4.17 and 4.18), even though the expression plasmids employed are functional (Figure 4.16).

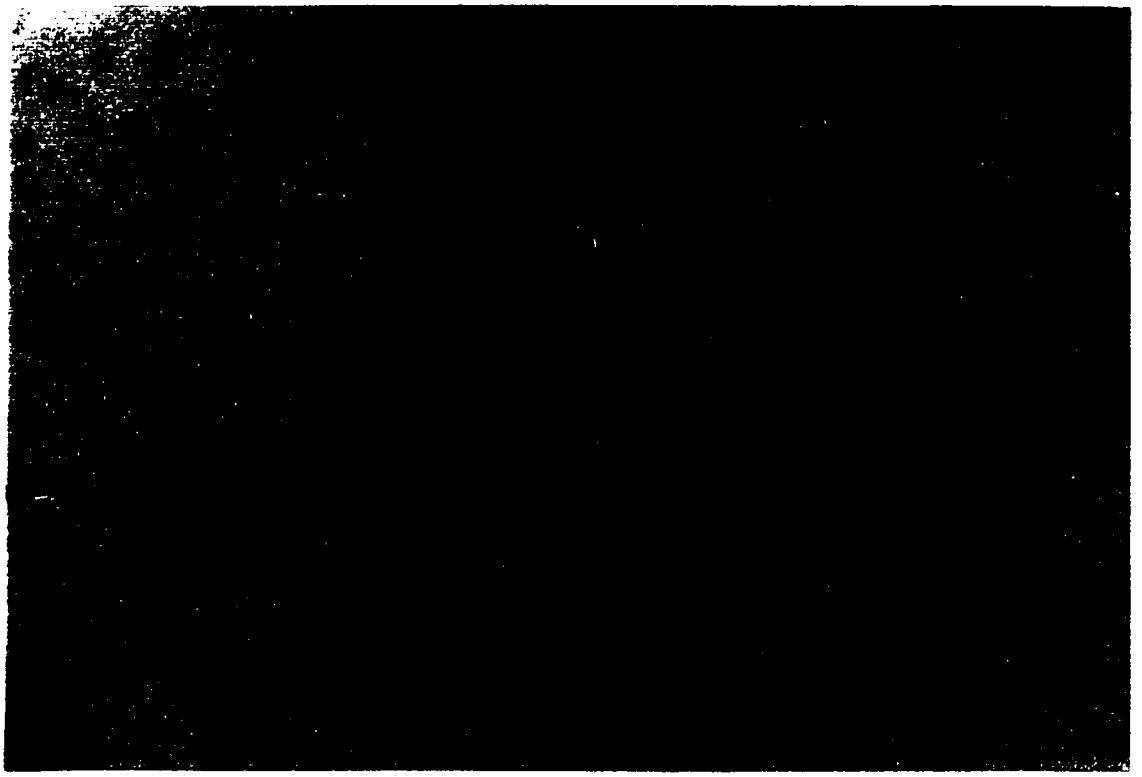


Figure 4.15 X-gal staining of term trophoblasts transfected with a β-galactosidase expression plasmid. Purified trophoblasts from term placenta were transfected with the β-galactosidase expression plasmid pcDNA3/Zeo/nlacZ using calcium phosphate-DNA coprecipitation. The control consists of cells that were mock transfected (no plasmid DNA). Cells were fixed on day 3 and stained with X-gal as described in Materials and Methods. Result shown is representative of two separate experiments.

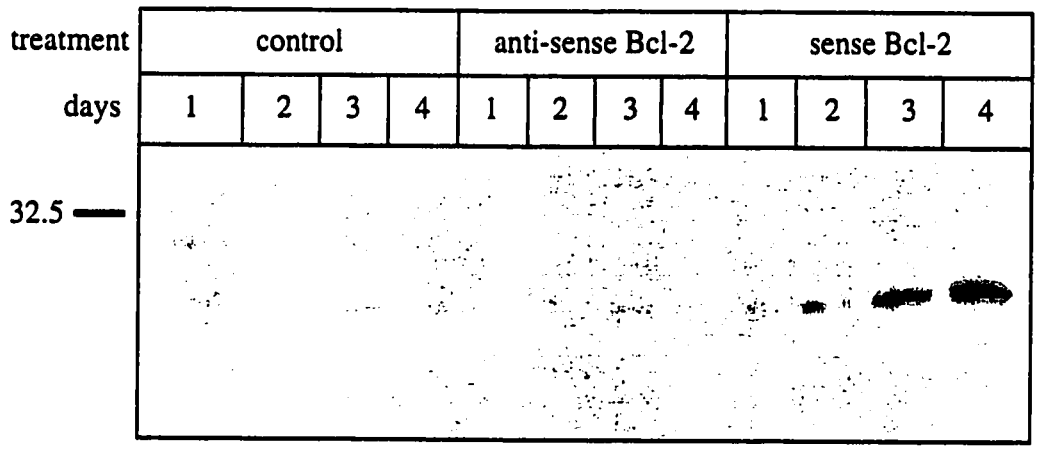


Figure 4.16 Immunoblot analysis of Bcl-2 protein expression in BeWo cells transfected with Bcl-2 expression plasmids. Confluent BeWo cells were transfected with antisense Bcl-2 expression plasmid pR509-8-18 or sense Bcl-2 expression plasmid pR509-8-45 using calcium phosphate-DNA coprecipitation. The control consisted of cells that were mock transfected (no plasmid DNA). Cells were lysed on the days shown and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein are loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size marker in kDa is shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein. The data shown are representative of three separate experiments.

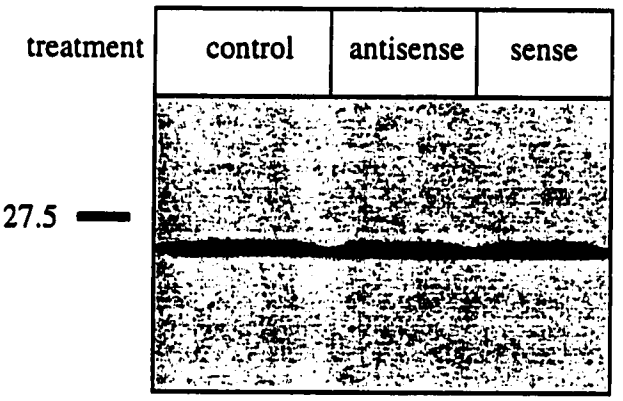


Figure 4.17 Immunoblot analysis of Bcl-2 protein expression in term trophoblasts transfected with Bcl-2 expression plasmids. Purified trophoblasts from term placenta were transfected with antisense Bcl-2 expression plasmid pR509-8-18 or sense Bcl-2 expression plasmid pR509-8-45 using calcium phosphate-DNA coprecipitation. The control consisted of cells that were mock transfected (no plasmid DNA). Cells were lysed on day 5 and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein were loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bcl-2 antibody (Dako) as described in Materials and Methods. Molecular size marker in kDa is shown on the left; the protein bands correspond to the 26 kDa Bcl-2 protein. The data shown are representative of five separate experiments.

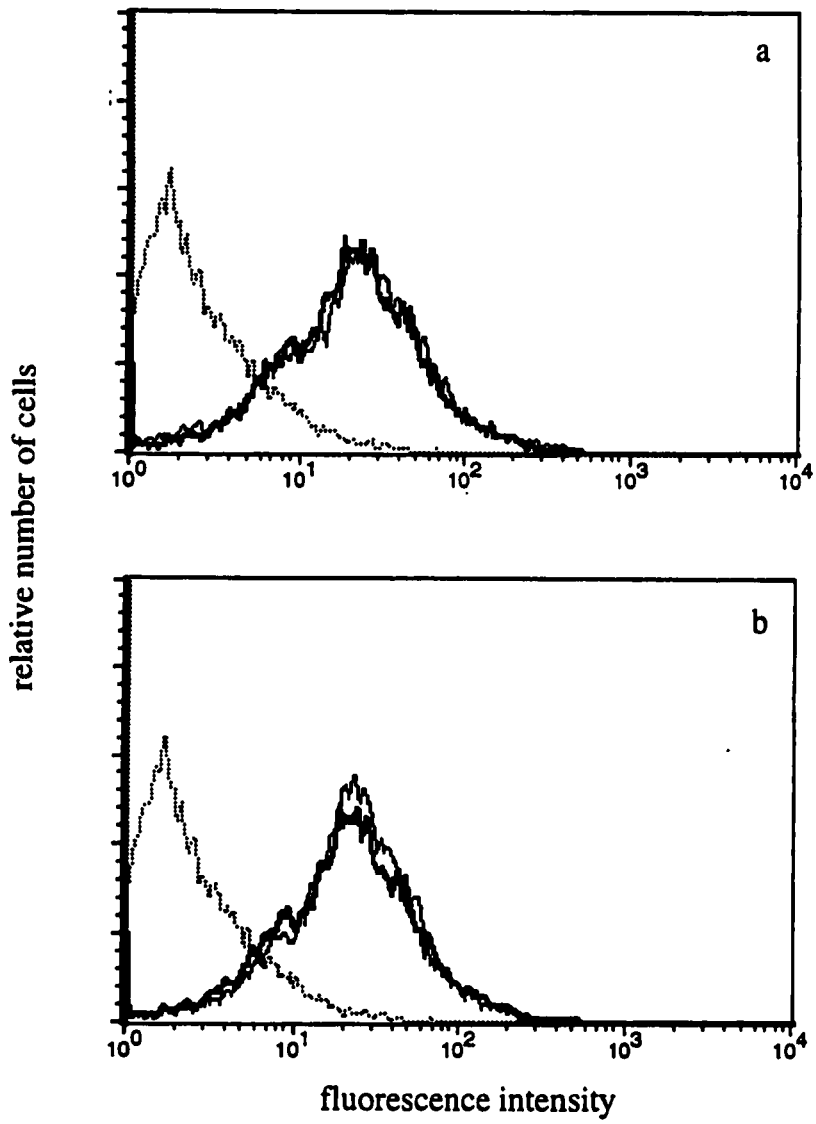


Figure 4.18 Flow cytometric analysis of Bcl-2 protein content in term trophoblasts transfected with Bcl-2 expression plasmids. Purified trophoblasts from term placenta were transfected with either antisense Bcl-2 expression plasmid pR509-8-18 or sense Bcl-2 expression plasmid pR509-8-45 using calcium phosphate-DNA coprecipitation. The control consisted of cells that were mock transfected (no plasmid DNA). On day 5 post-transfection, cells were immunofluorescence-stained with either monoclonal mouse anti-human Bcl-2 antibody (Dako) conjugated to FITC or FITC-conjugated isotype control antibody (Dako). a) FACS profile comparison of mock transfected cells (thin line) and sense Bcl-2 transfected cells (thick line). b) FACS profile comparison of cells transfected with sense (thick line) and antisense (thin line) Bcl-2. The dotted line represents antibody isotype control in both panels. The data shown are representative of three separate experiments.

CHAPTER 5

DISCUSSION

Vital to fetal development is the well being of the placenta. Besides providing nutrients to the fetus, this extraembryonic organ also acts as barrier against the maternal immune system and pathogens in the maternal circulation. Trophoblasts, being located at the fetal-maternal interface, are the cell type that carry out these functions. Pregnancy complications often results when these cells are damaged.

One type of damage is apoptosis. It has been found that TNF-α induces apoptosis in trophoblasts and that IFN-γ enhances cell death to 40-50% without being cytotoxic itself (Yui *et al.*, 1994 b; Figure 4.8). This apoptosis is suppressed by EGF and the glucocorticoid dexamethasone (Yui, 1994; Garcia-Lloret *et al.*, 1996; Figure 4.8). As these cytokines are found in non-pathological placentas as well (Bulmer *et al.*, 1990; Chen *et al.*, 1991; Paulesu *et al.*, 1994), they are likely to play a role in regulating trophoblast growth and placental villi remodeling in normal physiological conditions. TNF-α might facilitate the removal of unwanted trophoblasts by inducing their apoptosis, while EGF keeps the destructiveness of TNF-α in check and promotes new cell growth and differentiation (Yui, 1994). However, when TNF-α is present at excessive levels, pathological conditions could develop. There is extensive evidence on the correlation between TNF-α and pregnancy complications. These include pre-term labor pathogenesis (Casey *et al.*, 1989; Romero *et al.*, 1989; Hillier *et al.*, 1993), spontaneous fetal resorption (Tangri and Ragupathy, 1993), and intrauterine growth retardation (Heyborne *et al.*, 1992). On the other hand, cytokines such as EGF and GM-CSF are beneficial to placental health (Morrish *et al.*, 1987; Chaouat *et al.*, 1990; Loke *et al.*, 1992; Maruo *et al.*, 1992; Bass *et al.*, 1994; Fondacci *et al.*, 1994; Garcia-Lloret *et al.*, 1994).

The focus of this thesis was to understand the mechanisms of TNF-α/IFN-γ-induced apoptosis and EGF-mediated protection from such cell death. The

objective was to find a connection between apoptosis in trophoblasts and the anti-apoptotic protein Bcl-2. Bcl-2 has been shown to confer resistance against programmed cell death in many cell types. It does not have transforming capability. Instead it aids in cell survival by suppressing apoptosis. There is evidence that Bcl-2 counters TNF-α-induced apoptosis in various cell types. Recent studies show that Bcl-2 inhibits, albeit indirectly, caspases (cysteine proteases which are intimately associated with the execution of apoptosis), including those induced by TNF-α. These observations were the basis of my hypothesis that Bcl-2 is down-regulated during TNF-α/IFN-γ-induced trophoblast apoptosis in order to ensure the continuation of the cell death program. Conversely, EGF is proposed to up-regulate Bcl-2 to counter the effect of TNF-α/IFN-γ.

This hypothesis was tested using immunoblotting analysis (Figures 4.9 and 4.10). Trophoblasts cultured in medium supplemented with EGF, TNF-α/IFN-γ, or EGF/TNF-α/IFN-γ contained the same amount of Bcl-2 protein as cells cultured in medium alone. The same situation was observed in trophoblasts from first trimester and term placentas. TNF-α/IFN-γ-mediated apoptosis in trophoblasts can be enhanced in the presence of a sublethal dose (0.3 μg/ml) of cycloheximide (Yui, 1994). However, at this concentration of cycloheximide, Bcl-2 protein level was unaffected, implying that TNF-α and cycloheximide use a Bcl-2-independent pathway to affect trophoblast survival. Therefore, the hypothesis was not supported.

TNF-α/IFN-γ did not induce trophoblast apoptosis by lowering Bcl-2 content, nor did EGF protect cells from apoptosis by up-regulating Bcl-2. Bcl-2 protein levels remained stable independently of the extent of the cell death program. One interpretation is that Bcl-2 is not involved with pathways induced by EGF and TNF-α. Alternatively, Bcl-2 could be directly involved with EGF and

TNF-α pathways, but its levels are simply not manipulated by the cytokines to affect cell survival. The trophoblast's fate would thus be determined by the amount of Bcl-2 it expresses: cells with more Bcl-2 protein would be less likely to undergo TNF-α-induced apoptosis than cells with less Bcl-2.

Indeed, the latter scenario was found to be the case by analyzing the fate (life or death) of a population of trophoblasts divided into high and low Bcl-2 expressing subpopulations (Figure 4.14). The division and the fate were both determined by flow cytometric analysis. A cell was defined as apoptotic if it contained less DNA than 95% of the (healthy) cells, which have 2n DNA content. The FACS profile of Bcl-2 protein content was divided into approximate halves (high and low) at the peak staining intensity where there is little overlap with non-specific staining. This division of trophoblasts into high and low Bcl-2-containing subpopulations correlated with the degree of apoptosis. It was found that the degree of cell death in high Bcl-2 expressors was low and uniform (4-6%) even in the presence of TNF-α/IFN-γ (Table 4.1). In contrast, low Bcl-2 expressors had a higher frequency of apoptotic cells: cell death increased from 6% in control cells to 21% in TNF-α/IFN-γ-treated cells. This implies that trophoblasts with low Bcl-2 content were 3.7 times more likely to undergo inflammatory cytokine-induced apoptosis than cells with high Bcl-2 content (Table 4.2). Therefore, even though Bcl-2 was not modulated by cytokines that regulated trophoblast survival, its endogenous expression levels determined the fate of the cells when they were under assault by TNF-α.

TNF-α may either inactivate Bcl-2 protein function or activate proteins that oppose the action of Bcl-2. One such protein is Bak, an apoptosis-promoting homologue of Bcl-2 (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995). It was also expressed in the trophoblasts (Appendix). Like Bcl-2, its protein level remained unchanged when stimulated with EGF or TNF-α/IFN-γ. Whether

Bak is involved in TNF-α-mediated apoptosis in trophoblasts or if heterogeneity exists in protein expression is unknown. If either Bak or Bcl-2 are affected by TNF-α, it is most likely to be in protein activation instead of expression regulation. TNF-α signalling would trigger their activation or inactivation, and the relative amounts of each activated protein would determine the outcome of either death or survival. Alternatively, the action of TNF-α might be carried out by other, as yet unidentified, cell survival proteins in the trophoblast.

Although the immunoblot analyses in this thesis have shown little variation in Bcl-2 protein levels under different conditions, it is important to realize that the method measures the population as a whole. Subtle heterogeneity in Bcl-2 expression among trophoblasts is unlikely to be discerned by immunoblot analyses. Flow cytometric analysis remains a more quantitative and reliable method for documenting differences among individual cells. The FACS profile of Bcl-2 protein content in trophoblasts resembled a normal distribution (Figures 4.7 and 4.14). Approximately half the cells fell below or above the norm (which is also the peak value). Distinct populations of cells with higher or lower Bcl-2 content than the norm were absent. Thus, the expression of Bcl-2 is rather homogeneous. Despite this apparent homogeneity, there exists functional heterogeneity in Bcl-2 expression among trophoblasts. The above defined high and low Bcl-2 populations each spanned approximately five units of staining intensity. The fraction of cells with the highest Bcl-2 had about ten times more Bcl-2 than the lowest fraction and these latter cells appeared to be predisposed to apoptosis. This result is consistent with the immunohistochemical staining (Figure 4.6) where there were cells that stained strongly for Bcl-2 and others weakly. This points to the possibility that trophoblasts found in different regions of the placenta are biochemically different and perform distinct functions.

Throughout this thesis, EGF was found to have no effect on Bcl-2 expression. Although it protects trophoblasts from TNF-α/IFN-γ-induced apoptosis, it does not do so by up-regulating Bcl-2 protein levels (Figures 4.9 and 4.10). Its presence suppressed the degree of cell death from 21% to 13% in TNF-α/IFN-γ-treated low Bcl-2 expressing cells, while the percentage of apoptosis remained constant under the same circumstances in high Bcl-2 expressing cells (Table 4.1). This indicates that EGF protects trophoblasts by either enhancing processes downstream of Bcl-2 or by affecting a separate pathway parallel to the function of Bcl-2.

The glucocorticoid dexamethasone also rescues cells from TNF-α/IFN-γ-induced apoptosis (Yui, 1994). However, similar to EGF, dexamethasone did not provide protection to trophoblasts by increasing Bcl-2 protein levels (Figure 4.12). A recent finding suggests that dexamethasone modulates the effects of TNF-α by regulating TNF-receptor shedding in human airway epithelial cells (Levine *et al.*, 1996). Whether this occurs in trophoblasts awaits future investigations. Another cytokine that is beneficial to trophoblast proliferation and differentiation is GM-CSF (Loke *et al.*, 1992; Garcia-Lloret *et al.*, 1994), although it has no inhibitory effect on TNF-α/IFN-γ-induced apoptosis (Garcia-Lloret *et al.*, 1996). Likewise, GM-CSF had no effect on Bcl-2 protein expression.

These data support the notion that Bcl-2 expression is remarkably stable in trophoblasts. Both positive and negative regulators of trophoblast survival were unable to modulate its expression levels. Again, this either points to alternative pathways utilized by these regulators, or that they might influence the activation state or downstream events of Bcl-2. This warrants further examination.

The existence of heterogeneity in Bcl-2 expression among trophoblasts and its significance to cell survival suggests that increasing its expression would render the cells more resistant to TNF-α/IFN-γ-induced apoptosis and *vice versa*. Since

endogenous expression of Bcl-2 was found to be remarkably stable, one option was to transfect trophoblasts with exogenous Bcl-2 and overexpress the protein. The goal was to obtain a fraction of overexpressing cells and to test whether these cells were more resistant to TNF-α/IFN-γ-induced apoptosis. Conversely, if low Bcl-2 protein renders trophoblasts more susceptible to apoptosis, another option would be to decrease its expression with antisense to Bcl-2 transcripts and thereby make the cells more vulnerable.

The transfection method employed was calcium phosphate-DNA co-precipitation, which was used successfully in the transfection of HIV provirus (Kilani *et al.*, 1997) and β-galactosidase (Figure 4.15) into trophoblasts. This is a reliable way of introducing exogenous DNA into such cells. The transfection efficiency was estimated at 5-15%. The Bcl-2 sense and antisense expression plasmids were first transfected into BeWo cells (a transformed trophoblast cell line) to test their functionality. It was found that BeWo cells, which have a low Bcl-2 content, overexpress Bcl-2 after transfection with sense Bcl-2 (Figure 4.16). This indicates that the plasmid is functional. However, when the same experiment was repeated with trophoblasts, there was no observable change in Bcl-2 content as measured by immunoblotting analysis (Figure 4.17). It is possible that the 5-15% transfected and overexpressing cells were rendered undetectable by the existing endogenous expression of Bcl-2 using immunoblot. Therefore, the same experiment was analyzed with FACS. When these transfected cells were subjected to FACS analysis, the Bcl-2 protein content profiles of mock, sense, and antisense transfected cells overlapped (Figure 4.18). There was no evidence of small fractions of cells with higher or lower Bcl-2 content than the norm. Therefore, this approach was unable to alter the Bcl-2 protein levels in the trophoblasts, despite the fact that both the plasmid and the transfection method utilized were efficacious. The protein might be under tight expression control in these cells. This

possibility is consistent with the observations that Bcl-2 protein levels were unchanged by various external stimuli.

Previous documentation of the endogenous expression of Bcl-2 in the placenta has been limited. The few studies which addressed this subject are conflicting. To resolve this controversy and to theorize on the role of Bcl-2 in the placenta and trophoblast, studies on the expression of Bcl-2 were performed. The first objective was to examine Bcl-2 expression as a function of gestation time. The approaches were designed to determine which placental cell types express Bcl-2 and to compare the protein's content between placentas of different gestational age. The second objective was to examine Bcl-2 expression as a function of the differentiation state of trophoblasts, namely cytotrophoblasts and syncytiotrophoblasts.

To achieve the first objective, placental tissue section staining of Bcl-2 was performed (Figure 4.1). This gave information on the expression of Bcl-2 *in vivo*. The results showed that strong Bcl-2 expression was limited to the trophoblasts while stromal cells expressed very little, if any, Bcl-2. This was confirmed by the report of LeBrun *et al.* (1993) and Lu *et al.* (1993) of similar finding. However, Kim *et al.* (1995) have found Bcl-2 in other villous cell types such as villous mesenchymal cells and capillary endothelial cells too. The frequencies and staining intensities of these events were not mentioned in their study, nor was reproducibility and the antibody isotype controls shown. The difference between these two studies is therefore likely a matter of the frequency of occurrence and quality of staining. For Figure 4.1, six different placental sections were examined with no evidence of strong Bcl-2 staining in cell types other than trophoblasts. Because of the reproducibility of the current data, it was concluded that the strongest Bcl-2 expression was found in the trophoblasts.

Bcl-2 was found to be stained equally intensely in sections of both first trimester and term trophoblasts. In order to obtain a more quantitative analysis of Bcl-2 content, immunoblot analysis was performed on freshly isolated cytotrophoblasts from first trimester and term placentas (Figure 4.2). It was found that the expression level was the same in cytotrophoblasts isolated from both types of placentas. This is contradictory to the finding of Kim *et al.* (1995) that the Bcl-2 protein is undetectable using immunoblotting analysis in the placenta after 33 weeks of gestation. This discrepancy is likely due to the fact that they have used cellular extracts of whole placentas for their study. Cytotrophoblasts only constitute a small fraction of the total mass of the placenta, particularly at term. This is due to the fact that cytotrophoblasts stops proliferation after the second trimester (Weinberg *et al.*, 1970; Fox, 1978; Arnholdt *et al.*, 1991). As gestation time increases and the placenta grows, the ratio of the cytotrophoblasts to placental mass decreases. Therefore, it is technically incorrect to compare whole placental cell extracts from placentas of different gestational ages. In Figure 4.2, only purified cytotrophoblasts are compared. From this it is concluded that Bcl-2 expression was constitutive in cytotrophoblasts regardless of developmental state of the placenta.

The second objective to be addressed was the expression of Bcl-2 as a function of differentiation states of the trophoblast. *In vivo*, cytotrophoblasts continuously fuse with an overlying syncytialized layer of syncytiotrophoblasts. This process can also be observed *in vitro* in purified, cultured trophoblasts in the presence of EGF (Morrish *et al.*, 1987; Yui *et al.*, 1994 a; Garcia-Lloret *et al.*, 1994; Garcia-Lloret *et al.*, 1996; Figure 4.3). Using visual examination of placental tissue sections, LeBrun *et al.* (1993) have found that both CTs and STs express Bcl-2. However, they did not make a quantitative statement about the levels of expression between the two cell types. In contrast, Lu *et al.* (1993) have

found the protein in STs but not CTs. In the tissue section staining of Figure 4.1, the cytotrophoblast layer can be discerned from the overlying syncytiotrophoblast layer only in the first trimester placentas. This is due to the fact that cytotrophoblasts stop cellular division after the second trimester and the relative number of CTs falls dramatically (Weinberg *et al.*, 1970; Fox, 1978; Arnholdt *et al.*, 1991). Thus, there are fewer CTs in term than first or second trimester placentas. Visual examination of the first trimester placental sections showed that the strongest Bcl-2 staining was localized to the syncytiotrophoblast layer although the underlying cytotrophoblast layer still stained, but not as strongly. However, it is difficult to determine whether quantitatively STs contains more Bcl-2 than CTs quantitatively. The difference in staining intensity could be due to the size and shape of the cells. ST has more cytoplasm and the cell layer is larger and easily identifiable, while CT is smaller and the cytoplasm compacted. Therefore, at present, the difference in Bcl-2 expression between ST and CT *in vivo* is still equivocal.

Since *in vivo* data on trophoblast Bcl-2 expression as a function of differentiation state is inconclusive, *in vitro* studies were performed. Purified and cultured cytotrophoblasts were induced to differentiate into syncytiotrophoblasts with the supplement of EGF. Immunoblot analysis was then performed on cell lysates to examine Bcl-2 protein levels. It was found that undifferentiated CTs and differentiated STs from first trimester and term placentas expressed the same amount of Bcl-2 protein in culture (Figures 4.4 and 4.5). Therefore, Bcl-2 expression in trophoblasts in culture is constitutive regardless of the differentiation state of the trophoblast *in vitro*.

When trophoblasts were immunohistochemically stained for Bcl-2 in culture (Figure 4.6), it was observed that some cells stained more strongly than others. As time in culture increased, strongly staining cells coalesced into patches.

There are areas of intense staining surrounded by areas virtually devoid of strong staining. The same phenomenon is observed regardless of the developmental or differentiation state of the trophoblasts. In culture, the formation of cell monolayer involves trophoblasts moving towards each other and spreading out on the matrix. Cells which are alike might preferentially congregate together to give the patchy appearance upon Bcl-2 staining. This suggested Bcl-2 expression heterogeneity in these cells. However, this presents a conflict with the FACS profile of Bcl-2 protein content in trophoblasts, which is apparently homogeneous. Even though functional heterogeneity exists as demonstrated earlier, the discrepancy between these two physical measurements of Bcl-2 content needs to be accounted for. One possibility is that the apparent staining intensity is affected by the shape of the cell. A cell that is compact would appear to have stronger staining than a cell that is spread out, even if the two cells have the same protein content. Another possibility is the preferential loss of fragile cells during harvesting for FACS analysis.

In this thesis, the hypothesis that TNF-α/IFN-γ-induced apoptosis and EGF-mediated protection involves the modulation of Bcl-2 protein levels was not supported. Nevertheless, high Bcl-2 expression levels in a subpopulation of trophoblasts was shown to correlate with apparent resistance to TNF-α/IFN-γ-induced apoptosis. In contrast, cells with low Bcl-2 content were apparently more susceptible to cell death. The expression level is therefore heterogeneous among trophoblasts. Although EGF confers resistance to TNF-α/IFN-γ-induced trophoblast apoptosis, it does not alter Bcl-2 expression. Therefore, EGF and TNF-α/IFN-γ use alternative pathways to achieve their effect on the cells. Bcl-2 protein expression is remarkably stable in the trophoblasts. Its expression levels are not altered by various agents that influence the cells' survival. Within the placenta, Bcl-2 expression appears invariant with respect to the developmental and differentiation states of the trophoblast.

Further studies should concentrate on the significance of this expression stability in placental growth and well being. The heterogeneous nature of trophoblast requires more clarification. For instance, does this heterogeneity exist in syncytiotrophoblasts and cytotrophoblasts *in vivo*? The relationship between Bcl-2, EGF and TNF-α warrants more in-depth examination. What alternative pathways do EGF and TNF-α use to regulate trophoblast survival? Do the functional states of Bcl-2 and Bak change in response to these cytokines? Are there other Bcl-2 family members expressed in trophoblasts? Does the ratio of Bcl-2 to Bak change with gestation age? Perhaps more important is an understanding of the regulation of bcl-2 expression in trophoblasts and its physiological significance.

CHAPTER 6

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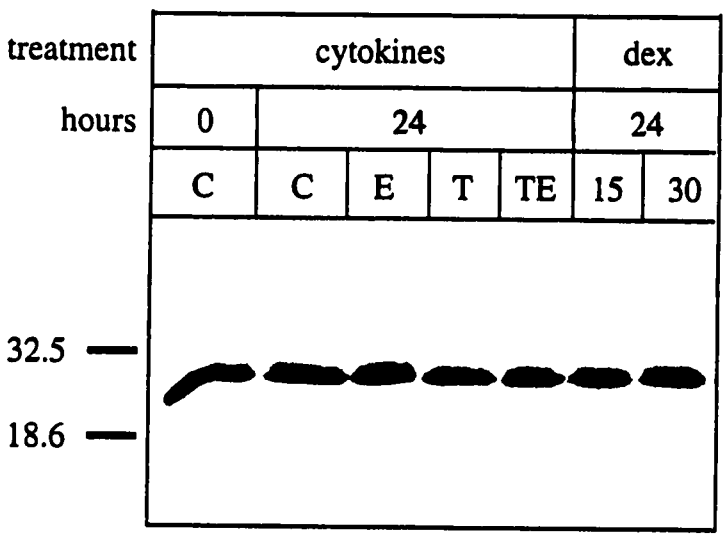
APPENDIX

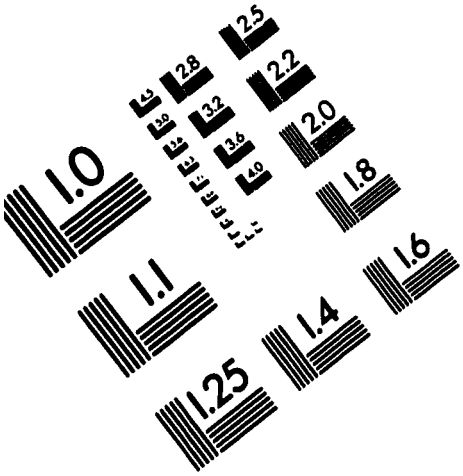
Preliminary study on Bak expression in placental trophoblasts

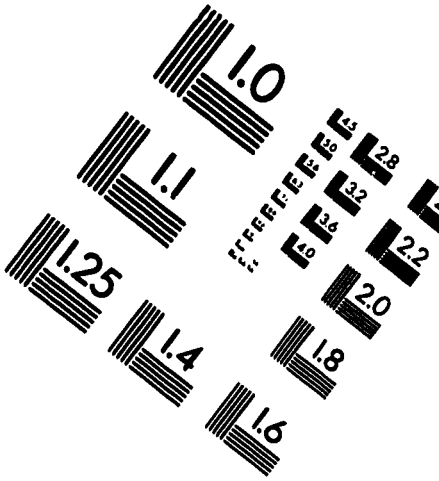
Although TNF-α does not alter the protein levels of Bcl-2 during trophoblast apoptosis, it might up-regulate proteins that could oppose the function of Bcl-2. One such protein is Bak, an apoptosis-promoting homologue of Bcl-2 (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995). In order to study Bak's expression in the trophoblast and its responses to agents that regulate trophoblast cell survival, purified trophoblasts from term placenta were cultured either in the presence of EGF, TNF-α/IFN-γ, EGF/TNF-α/IFN-γ, and dexamethasone. Immunoblot for the Bak protein was performed after 24 hours in culture (see figure on the next page). Bak is expressed in the trophoblasts. Like Bcl-2, its protein levels remained invariable under the conditions tested. Whether Bak is involved in TNF-α-mediated apoptosis in trophoblasts or if heterogeneity exists in protein expression is unknown at this point. Its presence however points to the possibility that the relative levels of Bcl-2 and Bak proteins a trophoblast possesses might determine the survival of the cell when it is challenged with factors such as TNF-α.

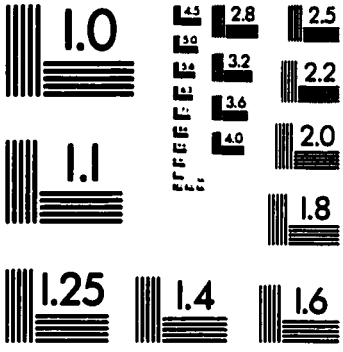


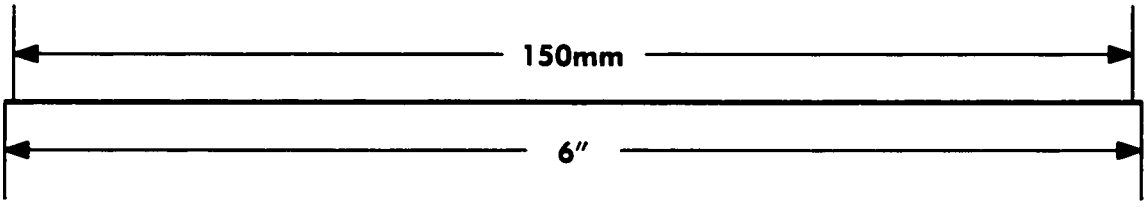
Immunoblot analysis of Bak protein expression in term trophoblasts cultured in cytokines or dexamethasone. Purified trophoblasts from term placenta were cultured in medium (10% FCS/IMDM) alone (control; C), medium supplemented with 10 ng/ml EGF (E), with 10 ng/ml TNF-α plus 100 U/ml IFN-γ (T), with both EGF and TNF-α plus IFN-γ (TE), or with 15 or 30 μM dexamethasone. Cells were lysed on the hours shown and lysates separated on a 4-12% gel as described in Materials and Methods. Equivalent amounts of protein were loaded in each lane. Immunoblot was performed using monoclonal mouse anti-human Bak antibody (CalBiochem) as described in Materials and Methods. Molecular size markers in kDa are shown on the left; the protein bands correspond to the 23 kDa Bak protein. The data shown are representative of two separate experiments.

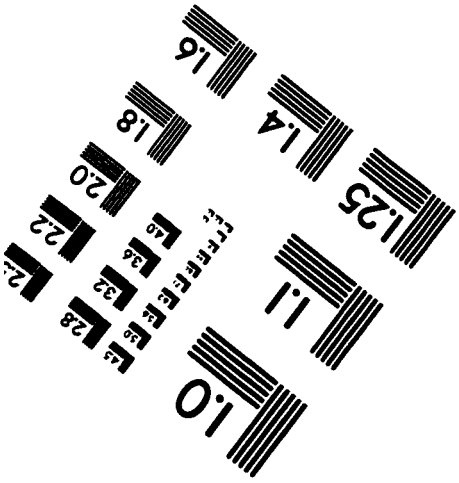
IMAGE EVALUATION
TEST TARGET (QA-3)

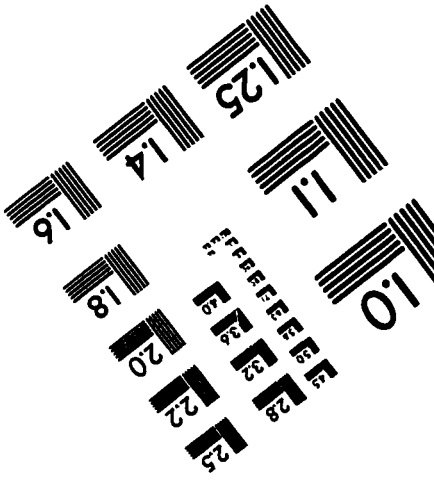












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