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The Effect of Interferon-α2b on Wound Contraction
and Scar Contractures

by

Bernadette Nedelec   ⓒ

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

in

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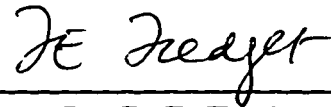
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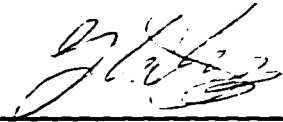
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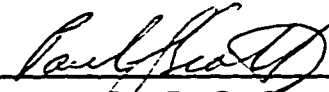
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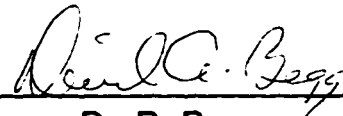
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DEDICATION

To those I love.

My family and friends,

without whom I could not survive.

ABSTRACT

Scar contraction following dermal injury is a leading cause of morbidity. The therapeutic use of interferon for fibroproliferative disorders has been suggested due to its antifibrotic properties. Assessment of the effect of interferon-α2b (IFN-α2b) on wound contraction was conducted within an *in vitro* model system, *in vivo* animal model, and by *in situ* analysis of human tissue. The fibroblast-populated collagen lattice (FPCL) simulates wound contraction. Using matched pairs of hypertrophic scar (HSc) and normal fibroblasts, exposure to IFN-α2b significantly inhibited contraction in a treatment time-dependent, serum sensitive manner. Comparison of HSc and normal fibroblasts revealed no significant differences in ability to induce contraction. Northern analysis revealed that IFN-α2b significantly down-regulated actin isoforms β and γ. FPCLs were stained with phalloidin which revealed morphologic alterations of the microfilaments following IFN-α2 b treatment.

To establish the effect of IFN-α2b on contraction of full thickness wounds *in vivo*, osmotic pumps loaded with interferon or saline were implanted in guinea pigs. Seven days later full thickness wounds were created. Comparisons indicated a significant reduction in wound contraction in the treated animals. Immunochemical analysis of the tissue indicated that although the relative amount of vimentin was increased after wounding, the myofibroblast-associated cytoskeletal proteins were not. Immunohistochemistry localized the expression of α-smooth muscle

actin (α-SMA) and lack of decorin, within the central region of the 21 day wound of the treated animal. Localization of apoptotic cells revealed an increase in the treated animal but this could not be localized to α-SMA staining.

The expression of α-SMA was quantitated in human burn wounds, HSc, normal scar and normal tissue. The percentage of myofibroblasts was significantly higher within HSc relative to normal scar or normal tissue. Staining for α-SMA was most intense in regions where abnormal collagen fibrils were present. The presence of myofibroblasts did not correlate with the severity of HSc. The total number of fibroblasts was twice that in normal tissue. Tissue sections obtained from patients who had received systemic IFN-α2b showed a reduction in myofibroblasts and total number of fibroblasts and an increased number of apoptotic cells.

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As I search for words that will adequately recognize all those who have supported me through my graduate studies, I am made acutely aware that none exist.

It is with profound gratitude and tremendous pride that I salute the people whose names follow as well as any I have inadvertently missed.

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ABBREVIATIONS

Aptex	3-aminopropyl-triethoxy-silane
BCA	bicinchoninic acid
BSA	bovine serum albumin
DAB	diaminobenzidine
DMEM	Dulbecco's Modified Eagle Media
EBD	epidermolysis bullosa dystrophia
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether) N, N, N', N'-tetraacetic acid
F-actin	filamenteous-actin
FBS	fetal bovine serum
FGF	fibroblast growth factor
FPCL	fibroblast-populated collagen lattice
G-actin	globular-actin
GAS	interferon-g-activated site
GITC	guanidinium thiocyanate
HSc	hypertrophic scar
ICSBP	interferon consensus sequence binding protein
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
IRF	interferon regulatory family
ISG	interferon stimulated genes
ISGF	interferon-stimulated gene factor
ISRE	interferon stimulated response element
mRNA	messenger ribonucleic acid
MANOVA	multiple analysis of variance
MDGF	macrophage-derived growth factor
NGS	normal goat serum
PBS	phosphate bufferred saline
PDGF	platelet-derived growth factor
PGE$_2$	prostaglandin E$_2$
PIP2	phosphatidylinositol 4,5-bisphosphate
PMSF	phenylmethylsulfonyl fluoride
PPI	polyphosphoinositides
RT	room temperature
SMA	smooth muscle actin
SSC	sodium chloride and sodium citrate
STAT	signal transducer and activator of transcription

TBSA	total burn surface area
TdT	terminal deoxynucleotidyl transferase
TGF	transforming growth factor
TNF	tumor necrosis factor
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
TRITC	tetramethylrhodamine isocyanate
TUNEL	transferase-mediated dUTP-biotin nick end labeling
VBSA	Vancouver burn scar assessment

CHAPTER 1:

GENERAL INTRODUCTION

1.1 INTRODUCTION

The development of hypertrophic scar following a burn injury is a common result following a deep partial thickness or full thickness injury. For a burn survivor, the restoration of function is intimately associated with the reduction of the scar contractures that occur over joint surfaces and mobile skin areas. Successful psychological adjustment is also partially dependent upon the physical disfigurement associated with scar contractures (1). One of the focuses of rehabilitation, either directly or indirectly, is the minimization and enhanced remodeling of hypertrophic scar (HSc). The better our understanding of the molecular, cellular and extracellular basis of the contractile process the more effective and efficient treatment interventions will become.

The clinical characteristics of HSc include red, raised, inelastic, itchy tissue that predominantly develops in wounds that require more than 21 days to heal (2). In HSc, collagen has been shown to be presnt as narrower fibrils arranged in nodular structures, rather than the fibres and fibre bundles seen in normal skin (13). Elevated levels of pro-$\alpha 2$(I) mRNA for collagen type I and secreted collagen in HSc fibroblasts compared to paired normal fibroblasts (7) suggests that excessive production of collagen is one of the contributing factors. This coupled with the reduced level of collagenase synthesis by hypertrophic scar fibroblasts (8) compounds the magnitude of excessive collagen production and the fibroblasts inability to normalize the collagen architecture. One of the fundamental differences between normal wound healing and HSc

is not simply the amount of collagen produced but rather the structurally superior fibre and fibre bundle organization (13). The stepwise sequences and essential collaborators that are required *in vivo* for the optimal progression of procollagen to fibres and fibre bundles are not clearly understood at present but it is becoming increasingly evident that various proteoglycans play a critical role (18). The excessive amounts of proteoglycans including versican and biglycan within HSc (16, 17) contribute to its rigidity and may frustrate fibroblasts efforts to realign collagen into fibres and fibre bundles. Conversely the lack of decorin (17), which has been implicated in collagen fibre formation (15), may further hamper normalization of the extracellular matrix architecture that correlates with supple yet durable tissue.

The elucidation of the pathology that contributes to the development of scar contractures has predominantly come from data which was generalized from wound contraction. The initial description of a fibroblast that possessed contractile properties can be traced back to the identification of a specialized mesenchymal cell found in granulation tissue which exhibited morphologic and biochemical features of both a fibroblast and a smooth muscle cell (5). The term "myofibroblast" was coined when it was shown that these fibroblasts possessed stress fibres and cell-to-cell contacts which were reminiscent of hemidesmosomes, making them smooth muscle-like fibroblasts (6). An alternative theory to account for contraction suggests that fibroblasts exert a traction force generated by the continual extension and retraction of filipodia in a treadmill fashion (10, 11) and that the myofibroblast appears

following the termination of dynamic contraction and locomotion (4, 14). Hence the predominant function of the myofibroblast may be to maintain the static equilibrium which has been achieved within the tissue and/or associated with programmed cell death (3).

Interferon-α2b (IFN-α2b) has been proposed as a potential therapeutic agent for the treatment of fibroproliferative disorders based on its ability to decrease fibroblast proliferation *in vitro* (12), to down-regulate the production of collagen and fibronectin (9, 19) and to enhance the production of collagenase (8). Prior to clinical application in the treatment of patients who have developed HSc, it is important to establish the effect that IFN-α2b has on contraction.

Chapter 2 provides a detailed literature review of topics that are pertinent to the subsequent chapters. Chapters 3, 4, and 5 describe the methodology, results, and conclusions of the *in vitro* experiments, the *in vivo* animal model experiments, and the human postburn scar evaluation respectively. Chapter 6 provides general discussion and conclusions from the culmination of the information described in the previous chapters and provides suggestions for the direction of future research.

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CHAPTER 2:

LITERATURE REVIEW

2.1 DERMAL WOUND HEALING

2.11 Introduction

Uncomplicated wound healing involves an orderly sequence of signals and responses resulting in protein production and cell proliferation. To achieve tissue repair platelets, leukocytes, fibroblasts, epithelial and endothelial cells are forced to interact outside of their usual domains with the ultimate goal being to re-establish normal tissue architecture. This complex series of events involves several overlapping phases including inflammation, proliferation, matrix deposition, contraction and remodeling. It must be kept in mind that this description in an oversimplification of a process that can be complicated by biological and biochemical factors or mechanical forces. These factors may cause a prolongation of a particular phase of wound healing, overlapping of phases or a return to an earlier phase as a result of their detrimental effects.

2.12 Inflammatory Phase

The repair process begins within minutes of tissue injury in order to achieve homeostasis. The initial injury causes structural damage to the vascular system which allows platelets to be released into the interstitial space. The platelets adhere to the exposed von Willebrand factor which is bound to the subendothelium and subendothelial collagen through their glycoprotein receptor GIb

(18, 158). In addition, binding and activation of coagulation factors at the platelet surface yields a membrane-bound prothrombinase complex which accelerates the generation of thrombin, itself a platelet activator (236). These interactions initiate platelet activation and coagulation which in turn forms the primary hemostatic plug. Released from the activated platelets are a variety of chemotactic and growth promoting cytokines (Table I) which will provide the stimulus required for the recruitment of white blood cells into the area of injury including neutrophils, monocytes and inflammatory phagocytes. As the stimuli which initiated clotting diminish, prostaglandins inhibit further platelet activation, aggregation and degranulation and plasmin and plasminogen activator begin to dissolve the thrombus (246).

The primary function of the inflammatory response is the removal of bacteria, debris and devitalized tissue and the production of appropriate signals that guide the subsequent tissue repair. The complex process of neutrophil emigration from the blood vessels involves the up-regulation of adhesion molecules (294) which mediate the attachment of leukocytes to the vessel wall. Neutrophil diapedesis is then facilitated by the release of a spectrum of vasodilating agents such as serotonin, histamine, bradykinin, arachidonic acid metabolites and nitric oxide (325). Once they are localized at the injury site neutrophils increase the expression and exposure of selective integrins. These integrins in turn function to enhance the neutrophils ability to act as the first line of defense by killing and phagocytosing bacteria and matrix proteins (125). In addition, neutrophils may play a key role in the production of

transforming growth factor-ß (TGF-ß), as they are the most abundant leukocyte cell type when TGF-ß levels are at their peak in a wound chamber model (56, 129).

Following establishment of hemostasis the primary function of monocytes/macrophages in the wound healing process is in the removal of pathogenic organisms, scavenging tissue debris and destroying any remaining neutrophils (17, 223). Monocytes are initially attracted to the wound bed by TGF-ß which has a chemotactic effect at low concentrations. Once they arrive at the wound site monocytes are activated to become tissue macrophages following exposure to bacterial products such as lipopolysaccharides and by high concentrations of TGF-ß (325). The critical role that macrophages play has been emphasized by experiments that have shown that neutrophil depleted animals heal normally (288) in contrast to macrophage depleted animals which exhibited delayed wound debridement and fibrosis (190). Since macrophages do not play a significant role in the production of extracellular matrix proteins, which defines fibrosis, the vital contribution of macrophages toward accomplishing this function is thought to be through the release of various cytokines (Table I) which are chemotactic for fibroblasts, angiogenic and potent immune modulators. If wound contamination occurs, the acute inflammatory phase will be prolonged with more extensive tissue damage as a result of attempts to rid the area of foreign substances and a subsequent delay in the formation of granulation tissue.

2.13 Proliferation and Matrix Deposition

The release and activation of cytokines and growth factors during the inflammatory phase are paramount to the second phase of wound healing which is characterized by angiogenesis, reepithelization, and activation of various fibroblast functions. Fibroblasts respond by migrating into the wound, proliferating, producing matrix components such as hyaluronic acid, collagen and fibronectin, facilitating wound contraction and reorganization of the extracellular matrix (ECM). The formation and function of these various tissue components are interdependent as the fibroblasts produce a substrate on which macrophages and new blood vessels can migrate into the wound and the neovasculature provides oxygen and nutrients for continued growth.

Angiogenesis is the growth of new vessels into the wound bed from blood vessels adjacent to the wound itself. It is stimulated primarily by the release of chemoattractants of endothelial cells by macrophages (175, 207), in addition to other factors such as heparin released from mast cells (15).

Reestablishing the integrity of the epidermis is paramount to wound healing as it provides a functional barrier which is one of the primary roles of skin. Epithelial cells respond to tissue injury by migrating in from the free edges of the wound and any remaining adnexal structures such as hair follicles, sebaceous and sweat glands (172). The time taken for this is the key difference between burn wounds and other forms of wounds making burn wounds more susceptible to infection and prolongation of the inflammatory phase

(142). The migration of epithelial cells into the wound occurs on a provisional matrix of fibrin and fibronectin and is stimulated by epidermal growth factor (EGF) (42). Once reepithelization has occurred they reestablish the basement membrane by producing laminin and type IV collagen (40). Following migration into the wound fibroblasts begin proliferating, becoming the predominant cell type responsible for matrix deposition and subsequent contraction and remodeling. Extracellular matrix components produced by fibroblasts that are important for wound healing include fibronectin, collagens, hyaluronic acid and proteoglycans. The early appearance of fibronectin in wound healing (184) facilitates its various functions including being a chemotactic factor for peripheral blood monocytes, a nonspecific opsonin for tissue debris, a substrate for fibroblast migration and a scaffold for new matrix formation (38). Type III collagen is detectable in wounds before type I (111) suggesting that type III fibrils may form the template for the deposition of the thicker type I fibres that are the predominant form in normal skin. The central role that cytokines and growth factors play in the stimulation of collagen and fibronectin production in wound healing at the level of gene transcription, translation and post-translation is beginning to be recognized (153, 257, 320).

Hyaluronic acid is the predominant glycosaminoglycan present early in healing wounds. Proteoglycans containing chondroitin sulfate and dermatan sulfate appear several days later (23).

An increase in the mast cell population in healing wounds is also thought to play an important role in wound healing. Histamine

and heparin produced by mast cells in the inflammatory phase of wound healing increase vasopermeability, neutrophil migration and plasminogin activation (141). The tumor necrosis factor-α (TNF-α) (118) produced by mast cells and fibroblasts may also affect fibroblast proliferation and collagen metabolism (74, 189, 210).

2.14 Contraction and Remodeling

The final phase of wound healing occurs after healing appears to be superficially complete. The scar undergoes a "maturation" process whereby the wound remodels into a more organized, elastic matrix. During this phase macrophages and fibroblasts continue to carry out extensive phagocytic debridement, a gradual loss of cells and vasculature occurs and the extracellular matrix protein profile changes in concentration and organization.

The process of wound contraction begins during the formation of granulation tissue as a beneficial component of wound healing which reduces the size of the wound. However, the role that wound contraction ultimately plays in wound closure is significantly less in humans relative to other species (122). It is accepted that fibroblasts are responsible for wound contraction but the factors affecting it and mechanisms involved have yet to be fully elucidated (refer to discussion below for further details).

In addition to contraction, remodeling involves the continued deposition and resorption of extracellular matrix proteins until mature scar is formed. This is a dynamic process whereby type III collagen is replaced by type I collagen and there are decreases in the

levels of hyaluronic acid and water. The balance of protein turn over is influenced by protein production and degradation. As previously indicated, fibroblasts are predominantly responsible for protein production and degradation is controlled by a group of enzymes including tissue collagenase, hyaluronidase and lysosomal proteases which can be produced by epithelial cells, fibroblasts, macrophages or leukocytes (211, 326).

2.15 Fibroproliferative Disorders

Burn wounds that require greater than 21 days to heal have been shown to develop hypertrophic scar (HSc) 78% of the time (64). The clinical characteristics of HSc include red, raised, inelastic, itchy tissue which often develops into contractures when over joints. The pathogenesis has yet to be well defined however it appears to be a result of excessive extracellular matrix production by fibroblasts present in the wound. The excessive production is likely influenced by high levels of fibrogenic cytokines such as TGF-ß and insulin-like growth factor (IGF-1) (see below).

The extracellular matrix components of the skin that have been implicated in HSc include collagen, elastin, glycoproteins, glycosaminoglycans and proteoglycans. In HSc, collagen has been shown to have narrower fibrils which are more widely spaced, rather than fibres and fibre bundles running parallel to the surface as in normal skin. Within nodules, which are never seen in normal skin, the collagen fibrils are more densely packed (196). Expression of pro-α2(I) mRNA for collagen type I was shown to be higher in HSc

fibroblasts compared to paired normal fibroblasts, as was secreted collagen (112). This finding led to further investigation at a tissue level which revealed significantly higher levels of mRNAs for type I and type III procollagen and TGF-ß in HSc compared to normal tissue obtained from the same patients. Interestingly the differences in HSc and normal were far more significant when mRNA extracted from tissue was compared relative to mRNA extracted from fibroblasts in culture suggesting that the local tissue environment significantly influences extracellular matrix production (113).

The temporary lack of elastin in scars has been implicated in the rigidity and inelasticity but this suggestion has not been rigorously pursued (280).

An increased concentration of glycoproteins was initially recognized by Shetlar and colleagues (282), with at least a portion of this increase being attributed specifically to fibronectin (173). The exact functional role that these glycoproteins play in the pathophysiology of HSc however has not been established (280).

Immunohistochemical analysis demonstrated an increase in the proteoglycans versican and biglycan in HSc yet a reduction in decorin, which was present throughout the normal skin (278). Interestingly TGF-ß was colocalized with decorin suggesting an active role of this cytokine in scar resolution. Chemical analysis of HSc, mature scar and normal skin showed 30% less hydroxyproline, 2.4-fold more uronic acid, and 12% more water in HSc compared to normal, however mature scar and normal skin were comparable for these three parameters. The content of decorin in HSc was markedly reduced whereas versican and biglycan were 6-fold higher when

compared to normal. The content of these three proteoglycans in mature scar was much closer to that of normal skin although some differences continued to exist (279). The abnormal proportions of these proteoglycans are implicated in the undesirable physical properties of HSc (for review of proteoglycans in HSc refer to reference number 280).

2.2 CYTOSKELETAL PROTEINS

2.21 Introduction

As discussed below it is generally agreed that contraction is mediated by the local cell population. The coordination and synchronization of the intracellular contractile elements and extracellular matrix proteins are processes that are still not well defined in non-muscle cells. To allow for a more detailed analysis of the sequence of events involved in contraction, it is necessary to have a basic understanding of the individual elements. To some degree actin, myosin, actin-associated proteins, microtubules, intermediate filaments, integrins and integrin-associated proteins have all been implicated. Hence the structure and function of those cytoskeletal proteins present in fibroblasts requires a more indepth review prior to discussing contraction.

2.22 Actin

There are six actin isoforms expressed in a tissue-specific, species-independent manner in mammals and birds (110, 297, 312, 314, 316). ß- and γ-cytoplasmic (or non-muscle) actins are found in all cells; however the other four actins (α- and γ-smooth, α-cardiac and α-skeletal muscle actin) are predominantly expressed in their designated muscle cells (90, 212). The cytoplasmic actins and γ-smooth muscle actin have been designated class I actins on the basis of sequence comparisons and the fact that they are encoded by

structurally related genes implying a common ancestral origin. The other three actin isoforms are designated class II actins (145). The actin isoforms exhibit >90% overall sequence homology, with the exception of the amino-terminus where the final 18 residues only show 50-60% homology (46, 199, 312-315). It is of interest to note that the variant amino-terminus region has been located within a major subdomain located on the surface of actin (29). The amino-terminal is also functionally important in that the methionine (and cysteine in the case of class II isoforms) is cleaved and the new amino terminus is then acetylated. This cleavage yields an actin which spontaneously assembles into filament bundles (50, 51, 258). Most actins are also methylated at His68 (29).

The molecular weight of the mammalian actins is approximately 42 kDa and contains approximately 375 amino acids (180). Dissociated actin subunits, refered to as globular actin (G-actin) are bound to either ATP or ADP and can polymerize into double-helical actin filaments (F-actin). The actin filaments are approximately 9 nm diameter helical, polar structures of uniformly oriented actin monomers whose formation is associated with rapid hydrolysis of ATP to ADP-P_i-F-actin and eventually ADP-F-actin (268). The polar structure of actin filaments results in two distinct ends-a relatively inert and slow-growing "minus" or "pointed" end and the fast-growing "plus" or "barbed" end. This terminology reflects the appearance of the filament when myosin is bound to it. The role of actin in nonmuscle cell contraction and motility was initially proposed by Loewy (197) when an extract similiar to "muscle actomyosin" was examined from the acellular slime mold

Physarum polycephalum. Initially it was speculated that the actin from muscle and nonmuscle differed in polymerization properties (181), but further investigation revealed that the regulation and perceived differences occurred as a result of interaction with other cellular components (180). Under identical experimental conditions (low temperature and physiologic ionic strength) skeletal muscle actin polymerization is approximately one log-order greater than that of non-muscle actin (117, 258). Monomer-binding proteins profilin and thymosin β_4 contribute to this difference in that they preferentially bind to non-muscle actin over muscle actin thereby maintaining them within the monomeric pool (186, 233, 330).

Fibroblasts generally express cytoplasmic β and γ-actin yet it has been demonstrated that under a wide range of pathologic conditions they also express α-SMA (Table II). All of the references listed in Table II imply a correlation between the expression of α-SMA and the deleterious symptomatology of the pathology being investigated. At this point however, the data supporting the cause and effect relationship between α-SMA expression and the functional alterations associated with these various pathologies are circumstantial. Evidence supporting the functionally diverse roles of actin isoforms has been accumulating in skeletal and smooth muscle cells. Localization studies and altered expression associated with differentiation suggest that stress fibres and myofibrils are predominantly composed of class II actins thereby contributing to the contractile potential whereas β-actin is associated with cell motility and cytoplasmic γ-actin with cytoskeletal arrangement (146). This type of molecularly based

research needs to be done in fibroblasts in relation to pathologic wound contraction.

2.23 Actin Binding Proteins

The discovery of actin binding proteins continues to be a dynamic, growing field of research with approximately 48 classes being identified at present (243). Briefly, the subsets that may be important in contraction, and hence will be expanded upon here, include monomer binding, capping, severing, crosslinking and lateral-binding proteins.

Actin monomer binding proteins such as profilin and ß-thymosin sequester actin monomers under resting conditions thereby preventing polymerization. Once activated, the critical concentration required for polymerization is theoretically lowered by the uncapping of the barbed ends or plus ends of filaments (see below). This effect can be amplified by monomer sequestration as it provides a reservoir of actin that may then be readily mobilized (35, 96). Of the monomer binding proteins, ß-thymosin plays the primary role of maintaining a pool of actin monomers that can be liberated for actin polymerization (265). Although profilin also functions as a monomer sequestering protein it has more elaborate roles suggesting that it is a mediator of membrane-cytoskeleton communication (203). During massive depolymerization, profilin acts as a nucleotide exchange factor which enhances the conversion of ADP-actin to ATP-actin, the latter being capable of polymerizing at a faster rate (116). Profilin also mediates cell membrane-

cytoskeletal communication by interacting physically with both actin and phosphatidylinositol 4,5-bisphosphate (PIP_2)(203).

The capping proteins bind preferentially or exclusively to the barbed or plus end of actin filaments thereby accelerating nucleation, inhibiting elongation, increasing the critical concentration and reducing the viscosity of F-actin, by generating shorter filaments (180). In non-muscle cells they are postulated to regulate the assembly and organization of actin filaments by binding to the barbed end in a Ca^{2+} independent manner. Once capping proteins have bound to actin monomers, new filament formation proceeds as their association prevents loss or addition of actin subunits at the barbed end. Additionally they may attach the actin filaments to the cell membrane or other cell structures thereby localizing new filament formation (53). Other proteins which cap the barbed or plus end of actin filaments have also been examined. gCap 39 or Cap G, which has particularly been examined in macrophages, dissociates from the actin filaments either when Ca^{2+} decreases or polyphosphoinositides (PPI) are increased, whereas gelsolin, which also functions as an actin filament severing protein, requires both a decrease in Ca^{2+} and increase in PPI prior to dissociation, facilitating polymerization (342). When fibroblasts were transfected with the Cap G gene to induce overexpression, the rate of translocation in response to chemotactic stimulus was increased, suggesting that new filament formation is functionally important for translocation to occur (299).

In addition to gelsolin, the actin filament severing family of proteins includes villin (predominantly found in epithelial cells), a

microvilli-specific isoform of gelsolin, and non-vertebrate severin and fragmin (341). In fibroblasts, electroinjection of free gelsolin increased migration (12). However, fibroblasts from transgenic gelsolin null mice showed a reduced rate of migration yet an enhanced ability to contract a collagen gel (337). Interestingly when the fibroblasts from embryonic gelsolin null mice were assessed for their ability to contract a collagen gel the wild-type fibroblasts had an enhanced rate of contraction.

The cross-linking proteins function to produce a three-dimensional network of actin filaments appropriate to the stresses being applied to the cell. This is accomplished by inhibiting the subunit addition or loss, stabilizing filament length and inhibiting treadmilling (ie subunit flux) (244). Filamin (actin binding protein-280) is the most abundant of these and appears to be responsible for the ability of the cell to maintain its form when subjected to sudden forces yet gradually deform with low load, sustained force (8). The amino-terminal actin binding domain is similiar to that of α-actinin and fimbrin. It produces perpendicular branching with actin filaments and accelerates the onset of polymerization. Sato and colleagues (267) demonstrated that α-actinin has a periodic distribution along stress fibers and overlaps with vinculin where the stress fibers terminate at the focal adhesions, where α-actinin also binds to the cytoplasmic domain of the β1-family of integrins (235). The properties of α-actinin have been shown to be different in non-muscle cells as compared to smooth and striated muscle. Within non-muscle cells the α-actinin cross links with actin filaments only in the absence of Ca^{2+} ions (73) which may be

accounted for by the fact that the nonmuscle isoform of α-actinin is an alternative splice variant of the smooth muscle isoform (10). Fimbrin also functions as a crosslinking protein but is specifically localized to the focal contacts (30).

Non-muscle tropomyosin has multiple functional properties which are associated with the expression of different isoforms. The low molecular weight isoforms have been localized to short actin filaments which appear in motile, invasive cells such as transformed fibroblasts, whereas the high molecular weight isoforms are within microfilament bundles and stress fibres. There is also some evidence that non-muscle tropomyosin stimulates myosin I and II in non-muscle cells but these investigations are limited (241). The actin severing capabilities of fragmin and gelsolin are hindered by the binding of tropomyosin which has been shown to protect the actin filaments thereby enhancing their stability (157, 332).

2.24 Myosin

The myosin superfamily has been classified into seven different types based on squence analysis (34). Myosin I molecules are single-headed with a short heavy chain that does not form filaments (182). Myosin II consists of two heavy chains that form a alpha-helical coiled-coil tail which folds to form the globular heads at the amino-terminal. The globular heads are associated with two light chains each, which therefore make it a hexameric protein. The individual subfragments of myosin II have the capacity to interact

with other myosin tails to form bipolar filaments and each head is able to bind with actin and exhibit actin-activated ATPase activity (328).

In order to generate tension for contractile activity the chemical energy of ATP must be converted into a biomechanical equivalent. Adelstein (2) summarized the fundamental difference between the actin-myosin relationship in smooth muscle and nonmuscle cells or fibroblasts as being one in which actin within these cells cannot be activated unless the light chain of myosin has been covalently modified by myosin light chain kinase phosphorylation at two specific serine residues (3).

The enzymes that catalyze the phosphorylation and its reversal are myosin light chain kinase and phosphatase. The effect that phosphorylation has is two fold. First a conformational change of the myosin head exposes the actin binding site hence converting it to its active form. Second it allows the myosin molecules to interact by releasing the "sticky patch" of the myosin tail from the head of the molecule. This conformational change is an important factor in myosin aggregation, shape and ability to self-assemble as dephosphorylation produces freely soluble molecules (8).

Regulation of myosin light-chain kinase is affected by free calcium concentration which is mediated through the protein calmodulin. When intracellular calcium concentrations rise above approximately 10^{-6} M, all four binding sites of calmodulin are occupied by calcium, this subsequently activates myosin light-chain kinase, which in turn phosphorylates one of the myosin light chains, converting it to its active form, thereby allowing it to interact with

actin. The phosphate groups required for phosphorylation of myosin are bound to myosin light-chain kinase, following its interaction with calmodulin, by cAMP-dependent kinase. If cAMP-dependent kinase phosphorylates myosin light-chain kinase in the absence of calmodulin, it decreases its activity (2).

The function of myosin that has been most widely investigated in non-muscle cells in its role in cell motility. This motility can take the form of movement within the cell of organelles or vacuoles, changes in the shape of the cell particularly during cell division and migration of a cell across a substratum. The form of motility of interest in this discussion however is that of cell motility on a substrate and the forces applied to extracellular matrix.

In *Dictyostelium ameba* myosin I may play a role in cell locomotion. Genetic studies suggested that it was not required for normal function but this is probably due to partial or complete functional redundancy of the different isoforms (163, 303). Localization of myosin I in fibroblasts in response to wounding suggests that it is most involved in the extension and/or retraction of protrusions at the leading edge and the transport of vesicles. Myosin II was colocalized with actin along transverse fibres in the lamellae of migrating cells and concentrated at the centre of the cell during contraction. The functional attributes of myosin II were therefore thought to be the generation of force to separate cells, maintenance of the direction of locomotion, maximization of the rate of locomotion and/or aid in the delivery of cytoskeletal/contractile subunits to the leading edge (48).

In an attempt to establish the functional importance of phosphorylation, Adelstein (2) notes that some investigators have found that myosin phosphorylation correlates with the development of tension but not its maintenance. Using electroinjection techniques, Wilson and colleagues (336) concluded that myosin II plays a central role in macrophage motility, as a change in myosin light chain kinase activity or myosin light chain phosphorylation impaired motility.

2.25 Intermediate Filament Proteins

The term intermediate filament is derived from the fact that their diameters are 10 nm which is smaller than that of microtubules (25 nm) and larger than actin filaments (9 nm) (100). As opposed to microtubules and actin filaments which are assembled from globular protein subunits, intermediate filaments are composed of fibrous proteins. Sequence analysis and structural comparisons have identified five types of intermediate filament proteins: I) type I keratin, II) type II keratin, III) vimentin, desmin, glial fibrillary acidic protein, peripherin, IV) neurofilament, α-internexin and V) lamin. More recently nestin and filensin have also been assigned to the intermediate filament protein superfamily but do not exhibit enough sequence or structural homology to be assigned to one of the five major types (105).

The secondary structure of intermediate filaments is common to all identified members of the intermediate filament superfamily. They all contain a long α-helix which dimerizes to form a coiled-coil

nearly 50 nm long which is flanked by nonhelical N- and C-terminal sequence. The α-helix is subdivided into three segments which are linked by short nonhelical sequences. Between the various types of intermediate filament protein the variability in amino acid sequence and length is concentrated within the nonhelical head and tail sequence. Filament assembly occurs spontaneously when anti-parallel dimers align in a head-to-tail fashion forming linear polymers refered to as protofilaments. Four protofilaments align to produce the protofibril which is approximately 10 nm in diameter (105).

The functional role of intermediate filament proteins was thought to be an intracellular structural one since they were extremely stable when treated with high salt buffers and refold spontaneously in near-physiological buffers. They were also thought to be static cytoskeletal elements in view of their unusual stability but the role of phosphorylation in the dynamics of intermediate filament organization is gradually being uncovered (91, 290). The fact that several cultured cell lines and tumors exist which totally lack intermediate filament proteins and that no detectable damage or functional disturbances were observed when intermediate filament proteins were immunoprecipitated within living cells following the injection of antibodies, has further complicated the assignment of distinct cellular function (100). Transfection experiments however have clearly been able to show that filament formation requires an intact α-helical rod domain and N- and C-terminal domains, the latter of which may also be important in the initiation and assembly of intermediate filaments (6, 7, 54, 200

318). As an extension of the transfection experiments, naturally occuring mutations of intermediate filament proteins have been identified in association with epidermolysis bullosa simplex, hyperkeratosis and palmarplantar keratoderma (105). However vimentin null mice (vimentin is the intermediate filament protein expressed in mesenchymal cells including fibroblasts) interestingly demonstrated no obvious phenotype (47) which suggests that although mutations impair normal function of cells, the absence of vimentin is readily tolerated by cells.

2.26 Microtubules

With few exceptions, microtubules *in vivo* are constructed of thirteen protofilaments that contain α,β tubulin heterodimers aligned in a polar fashion. The density of microtubules is greatest around the nucleus where they grow in a GTP dependent fashion from specific microtubule organizing centres resulting in hollow 25 nm diameter cylinders (41). γ-tubulin has been identified more recently and localized to the microtubule organizing centre where it is believed to play a role in microtubule subunit initiation (14).

Microtubules interact with a collection of associated proteins (Microtubule Associated Proteins or MAPs) that function to regulate their assembly and disassembly. MAPs specifically interact with the carboxy-terminal domain of tubulin through their binding domain and appear to interact with actin filaments and intermediate filaments through their projection domain, although the biochemical and functional basis of the latter interaction has yet to be fully

elucidated. Microtubule motor proteins such as kinesin and cytoplasmic dynein serve as the driving force for microtubule-associated intracellular traffic (201). Functions that have been attributed to microtubules include maintenance of cell shape, chromosomal translocation during mitosis and the transport of vesicles, cell organelles and extracellular macromolecules. Microtubules appear to work in concert with actin filaments to produce migration in fibroblasts and provide the transport system for endocytotic cycling during cell migration (41).

Since microtubules exist in a state of dynamic instability, they are sensitive to drugs that bind to tubulin. Colchicine, colcemid, nocadazole, vinblastine and vincristine depolymerize microtubules, the former three by inhibiting the addition of tubulin molecules and the latter two by the formation of paracrystalline aggregates of tubulin (206). Taxol on the other hand promotes microtubule assembly (or inhibits disassembly) (269). Experimentally these drugs have been exploited to assist in descriminating the functional roles of microtubules.

2.27 Integrins and Integrin-Associated Proteins

Integrins are a family of proteins that provide the link between the cytoskeletal proteins within a cell and their external environment. These interactions can take place between cells and extracellular matrix proteins or other cells and involve a complex of intracellular proteins that have been referred to by a number of terms such as adhesion plaques, focal contacts or focal adhesions.

The integrins are transmembrane glycoproteins which consist of an α and β subunit that combine in a variety of patterns in order to alter their adhesion specificity (55, 63). The integrin subunits have received a significant amount of attention in relation to wound healing. $\alpha_5\beta_1$ was thought to be important as a result of its interaction with the RGD sequence of fibronectin (261). However its role in cell-collagen interaction is unclear since fibronectin is not required for fibroblasts to attach and spread on collagen fibrils or to contract collagen gels (127, 128, 131, 132, 304). More recently it was demonstrated that it is the $\alpha_2\beta_1$ integrin that is required for fibroblast contraction of a type I collagen matrix (174, 272).

Intracellularly, integrins associate with a number of different proteins to produce focal adhesions. These include: talin, vinculin, α-actinin, tensin, focal adhesion kinase, paxillin and F-actin (55, 339). The complexity of the intermolecular interactions are still being uncovered with strong evidence to support the concept that cell-matrix interactions are capable of both receiving and generating signal information which leads to cellular responses such as conformational changes, loss of adhesion leading to apoptosis, proliferation, motility, differentiation, integrin activation and intracellular signalling (63, 260, 262, 300, 339). The associated F-actin may take the form of stress fibres which includes α-actinin, myosin, tropomyosin, myosin light chain kinase and caldesmon or the more dynamic actin filament network associated with the leading edge of motile cells and filopodia both of which are more actively cycling actin monomers (202, 300, 333, 344). The signals which determine the cell morphology appear to be mediated through the

small GTPases Rac, Rho, and Cdc42, all of the Rho GTPase family of proteins. Activation of Rac triggers lamellipodia formation and focal complexes as integrins become clustered. Activation of Rho results in actin reorganization into bundled stress fibres with associated large focal adhesions. Activation of Cdc42 creates actin filament rich filopodia protrusion from the cell body (202, 228, 300, 344).

2.3 CONTRACTION

2.31 Introduction

Wound contraction is a double edged sword in humans. It contributes to wound closure, albeit to a much greater extent in animals than in humans (34, 248), which is a beneficial process in the case of a large burn injury. Several weeks or months later what appears to be the same process can lead to extensive functional and cosmetic problems. The various components and factors that are required for the development of contracture formation associated with HSc are currently not well understood.

2.32 The Myofibroblast

Myofibroblasts have been proposed as a sub-type of fibroblasts that are responsible for the contraction of healing wounds. The myofibroblast was initially defined as a specialized mesenchymal cell found in granulation tissue which exhibited morphologic and biochemical features of both a fibroblast and a smooth muscle cell (106). The term "myofibroblast" was coined when it was shown that strips of granulation tissue contracted in vitro when treated with smooth muscle stimulants (205). Electron microscopic analysis revealed that the myofibroblast possessed an extensive cytoplasmic fibrillar system (stress fibres), undulations of the nuclear membrane, abundant endoplasmic reticulum and peripheral attachment sites reminiscent of hemidesmosomes. Thus, the

myofibroblast was proposed as a fibroblast which becomes modified into a smooth muscle-like cell (107). From this data the hypothetical mechanism of wound contraction involves cell-to-cell contacts resulting in multicellular rearrangement of the surrounding connective tissue as a result of cell shortening (263). More recently the development of a monoclonal antibody which specifically recognized the α-smooth muscle isoform of actin (292) has been used to show that myofibroblasts express increased amounts of α-SMA, rather than β and γ-cytoplasmic actin isoforms, which are characteristically expressed by normal fibroblasts. The expression of α-SMA in fibroblasts has therefore come to define a myofibroblast. Myofibroblasts have been further divided into five phenotypes as defined by the variations of cytoskeletal expression: 1) expressing vimentin (V-cells), 2) co-expression of vimentin and desmin (VD-cells), 3) co-expression of vimentin and α-SMA (VA-cells), 4) co-expression of vimentin, desmin and α-SMA (VAD-cells) and 5) co-expression of vimentin, α-SMA, desmin, and smooth muscle myosin heavy chains (VADM-cells) (32, 37, 266). It has been suggested that V-type cells are present in early granulation tissue and are replaced by VA-type cells during the active retraction phase, whereas VADM-type cells may be present in HSc, liver cirrhosis, lung fibrosis and Dupuytren's disease (68). Using the fibroblast-populated collagen lattice (see below) it has been demonstrated that increased contractility correlated with higher concentrations of α-SMA expression in several primary cell strains (11). However, substantial variability among fibroblast strains, as discussed below, does not exclude the possibility that the differences in rate

of contraction could be attributed to other factors that were not assessed.

An alternative theory of contraction proposes that fibroblasts exert a traction force generated by the continual extension and retraction of filipodia in a treadmill fashion (137, 138) rather than by progressive shortening of their cytoskeleton. This theory is supported by the observations that stress fibres are not essential for motility and that cellular motility is associated with a more diffuse distribution of actin and myosin which is most consistent with continuous rearrangement of the cytoskeleton (146). The microfilaments that do form remain stationary as the nuclear region moves towards the anterior focal contacts and then decay, coupled with continual recycling of disassembled cortical actin to the dorsal lamellipodia (1).

Several pieces of evidence also argue against the specific role of the myofibroblast as the contractile cell in tissue. It has been demonstrated that myofibroblasts are prominent *in vivo* in the presence of mechanical tension yet in the absence of wounding (296) suggesting that there may be a feedback mechanism from the extracellular environment which induces the myofibroblast phenotype, rather than the myofibroblast specifically influencing the ECM. Also, Leavitt and colleagues (187) have demonstrated that transformed cell strains expressing α-SMA, phenotypically exhibited greater contact inhibition and lower invasive and motility capabilities (features required of fibroblasts during wound healing and contractile situations), as compared to those lacking α-SMA expression.

More recently it has proposed that the myofibroblast appears following the termination of dynamic contraction and locomotion (78, 259), which suggests that its function is to maintain the static equilibrium which has been established within the tissue. After examining fibroblasts using immunohistochemical techniques and electron microscopy, Singer and colleagues (289) found that the close relationship between the actin cytoskeleton and the fibronectin containing extracellular matrix was characteristic of differentiated fibroblasts which occurred when cells were growth arrested, immobile and well spread out on the substratum. Interestingly, examination of the time-course in open wound healing has shown that α-SMA is maximally expressed at 12-15 days and actin filaments at day 15-21, both of which correlate with the end of the most actively contractile period (61, 71). The correlation of myofibroblasts with apoptosis (programmed cell death) in an open wound healing model or following the application of a tissue flap (69, 109), suggests that α-SMA expression correlates more with the termination of a proliferative/migrational phase and characterizes a terminally-differentiated cell. In aortic smooth muscle cells a similiar correlation is seen where α-SMA and smooth muscle myosin heavy chains are expressed during quiesecent periods whereas nonmuscle actin and myosin are expressed when the cells are stimulated to migrate and proliferate (145, 255). In addition it has been shown that fibroblasts with strong fibrillar α-SMA staining in low-density cultures rarely incorporated 5-bromo-2'-deoxyuridine which indicates they are less proliferative (208).

One of the difficulties that arises, when trying to determine the impact of the various factors involved, is the heterogeneity of the pathologies being compared. For example granulation tissue and HSc represent very different microenvironments yet findings are often generalized from one setting to another. In addition, parallels are drawn between the organization and functional interaction of cytoskeletal proteins of a motile cell and that of a cell that is thought to be causing tissue contraction. These are potentially very different processes in that contraction may or may not require cell migration within the matrix. It has also never been shown that tissue shortening associated with HSc is not the result of an inability of fibroblasts to effectively organize the ECM proteins into an organized and flexible matrix as is seen in normal skin. Considering the accelerated rate and altered proportions of functionally important ECM proteins being produced this may warrant further investigation. It is possible that the rigidity of the tissue is the result of a lack of ECM reorganization hence scar contractures are actually the result of a lack of fibroblast mobility and matrix reorganization and not the other way around.

2.33 In Vitro Wound Contraction

As is obvious from the previous discussion, the processes of fibroblast mobility and contractility in vivo are complex. Since Bell and colleagues (19) description of the fibroblast- populated collagen lattice (FPCL), it has become a popular model system to simulate progressive cellular contraction of an extracellular matrix. The

process involved in setting up this model, requires the mixing of cells and soluble type I collagen then warming to 37° which facilitates polymerization of the collagen. The cells will attach to and rearrange the fibrils, thereby condensing the lattice (127). The size can then be measured as a function of time. If the number of cells within the lattice is increased or the concentration of collagen is decreased the rate of contraction will increase (19). This allows the assay to be adjusted so that the contractile period will plateau over a period of hours, days, or weeks. The advantage of this model is that it simulates connective tissue contraction during wound healing and hence allows for quantification of the influence of various components on the rate and extent of contraction. In addition, it more closely resembles the tissue equivalent than does monolayer culture on plastic and hence constitutes a more normal state of cell function.

The use of this model has expanded to include floating lattices (as described above), which reduce in diameter, attached lattices, which reduce in height, and stress relaxation where mechanical tension is allowed to develop over time and then the lattice is released from the plastic (126) resulting in an abrupt reduction in diameter. In the floating lattice, inhibitors of DNA synthesis such as hydroxyurea, mitomycin (79) and cytosine arabinonucleoside (19, 53) had no effect on contraction rates indicating that cell proliferation is not a significant contributing factor. In addition it has been shown that the proliferation of fibroblasts is repressed within the collagen lattice (33, 220, 226, 275) as the cells are arrested in G_0 when embedded within the floating lattice (179). This

is in contrast to the attached gels (220) and stress relaxed gels where they continue to proliferate until the point that they are detached (215). Conversely if an external force is applied to the floating lattices the cell proliferation rate will increase (159).

The FPCL has been used to evaluate the contractile activity of a number of different cell types but the discussion here will be limited to fibroblasts. Extensive research has been conducted to evaluate the effect of soluble factors using this model with the information falling into several different categories. One general grouping includes those factors that increase the contractile rate, such as transforming growth factor-ß (TGF-ß) (216, 250, 302), human platelet-derived growth factor (PDGF) -AB and -BB (39, 133, 224), sheep amniotic fluid (254), ß-aminopropionitrile (338), and an increase in serum concentration (33, 131, 298) which is possibly due to the presence of TGF-ß and PDGF. The rate of contraction can be decreased by heparin (130), rat wound fluid (253), corticosteroids (53), cortisol, ß-estradiol, dexamethasone (319), minoxidil (151, 338), and interferons (60, 115, 221, 264). Components that have been found not to have any effect include PDGF (216), cAMP (131), EGF and fibroblast growth factor (FGF) (216, 224). It has been suggested that the discrepancy when examining the effect of PDGF was as a result of varying experimental conditions where the contractile period in one experiment was over twenty four hours whereas the other extended over several days (132, 216). When treating the cells with various growth factors Nishiyama and colleagues (224) found that although several of them

induced a mitogenic response in culture, only PDGF stimulated growth when the cells were incorporated within a lattice.

It has been shown that protein synthesis is required for gel contraction since actinomycin, which inhibits mRNA synthesis and cycloheximide and puromycin which inhibit the translation of proteins, all inhibit contraction (79, 131). Collagen synthesis by fibroblasts incorporated within gels is significantly less than in the same cells grown as a monolayer on plastic with a lower rate of collagen degradation within the gel and the majority of the newly produced collagen remaining in solution (20, 209, 232). Cells within an attached lattice have a higher rate of protein production than floating gels until the point of stress relaxation which causes an abrupt reduction in type I procollagen and fibronectin (215). An analysis of labelling with ^{35}S-sulphate revealed that heparan sulfate and dermatan sulfate showed an increase rate of synthesis when cells are incorporated within a gel as compared to when grown on plastic. Hyaluronic acid was the main ^{3}H-glucosamine-labelled component from cells on both substrata, being produced in greater quantities when cells were grown on plastic rather than incorporated within gels (108) yet the exogeneous addition of hyaluronic acid, dermatan sulfate or fibronectin (129) did not have any effect on the rate of contraction.

Specific features of exogenously added extracellular matrix components have also been examined. The method used to extract the collagen was found to effect the rate and extent of contraction (338). If pepsin was used to extract collagen the rate of contraction was reduced since pepsinization removes the telopeptides which are

involved in collagen crosslinking. The use of type III collagen increased the rate and extent of contraction compared to type I, with type II being least readily contracted (77). Using Chinese hamster ovary cells transfected with the decorin gene to enhance the rate of decorin production, it was shown that the increased quantity of decorin produced by the cells inhibited contraction (25).

The integrity of the cytoskeletal proteins within the cell has been shown to be essential to the contractile process within the FPCL. Gel contraction was reduced with the addition of cytochalasin D (22, 131, 225, 305) and cytochalasin B (19, 85) which fragment the actin filaments. Colcemid (19, 22, 225), colchicine (79, 82) and nocodazole (225, 305), which disrupt the microtubules, also reduce the rate and extent of contraction by inhibiting the final elongation of the fibroblasts (305). Following the release of tension within the stress relaxation model there is a collapse of the microfilaments with a loss of visible bundles (95). Vesicles left behind by the cells contained actin, ß1 integrins and annexins II and IV (188). Although the physiological function of these vesicles has not been well established they probably represent sites at which the cells were attached to collagen fibrils that were released at the time of gel detachment and microfilament disruption.

Tomasek and Hay (305) summarized the morphological changes and cytoskeletal alterations in fibroblasts when populated in a collagen lattice, using electron microscopy and immunohistochemistry. The initially rounded cells gradually transform into a bipolar, elongated conformation by extending thin myosin-free filopodia. When grown on plastic the cells are flat and

broad with a ruffled border on the "upward-swinging leading edge". Stress fibers are characteristic of fibroblasts cultured on glass or plastic and actin is predominant in the microfilamenteous cell cortex and myosin in the cytosol of fibroblasts within collagen lattices. Tomasek also reports that the highly developed close or focal contacts of cells on a planar substrate are not present in lattices and the microtubules are radially distributed, as opposed to circular. The steps that they suggest fibroblasts progress through are 1) filopodial probing, 2) subsequent filopodial restriction to opposite poles of the cell, 3) extension of pseudopodia from these same cell poles and 4) finally cell elongation. From their research they conclude that an intact actin cytoskeleton alone is sufficient for the development of the bipolar shape but that both microtubules and actin microfilaments are required for the final elongation step. The ability of cells to fragment the initial actin microfilaments that stabilize the spherical scaffold is thought to be important in their ability to ultimately reorganize into the elongated morphology (227). Ehrlich and Rajaratnam (84) examined the location of fibroblasts with extensive cytoplasmic microfilaments and their possible function. By cutting the lattices into donuts and triangular wedges they localized such cells to the periphery of the lattice where active contraction had slowed or stopped and those fibroblasts with fine cytoplasmic microfilaments were localized to the centre where the active contraction was taking place. They therefore concluded that fibroblasts that are actively mobile create the contraction and myofibroblasts evolve once the active contractile period is completed.

Nishiyama and colleagues (225) summarized the kinetics of gel contraction by dividing it into three distinct phases. First a lag phase which occurs prior to any visible contraction. During this phase the cells are actively transforming from a spherical shape. They then extend pseudopodia which increase in length. Finally they assume a bipolar morphology. It is thought by several authors (19, 33, 65, 86) that these morphologic changes are essential if contraction is to occur. The second phase is a rapid contraction phase that can be extended over time by decreasing the number of cells and/or increasing the collagen concentration. The final phase is a slow contraction phase which may be a function of physical inability to compact the lattice further. It is emphasized by Nishiyama that the final slow contraction phase must be followed for a sufficient period of time as the contractile curves of the two treatment groups may ultimately meet. Exogenous factors may have an affect on one of the three phases of contraction and not another. If the effect of factors on the three phases are not separated out, specific correlations may be overlooked.

Studies with cell strains other than dermal fibroblasts have produced a variety of conclusions. Bellows and colleagues (21) compared monkey periodontal ligament fibroblasts, gingival fibroblasts, rat embryonic calvaria cells, porcine peridontal epithial cells and rat osteosarcoma cells, with the contractile rate decreasing in the listed order. The rat osteosarcoma cells did not contract at all, which the authors thought was a reflection of *in vivo* function of the various cell types. Finesmith and colleagues (97) examined rat granulation tissue at various stages and found that

cells from the older wounds (21 and 28 days) were better able to contract the lattices. bFGF was also shown to decrease the contraction rate at high concentration, and increase it at low concentration. TGF-ß increased the rate of contraction, as did TGF-α in old granulation tissue. An evaluation of the interaction between of TGF-ß and bFGF was also undertaken which revealed an alteration of TGF-ß effect. Pretreatment with bFGF followed by TGF-ß, enhanced contraction of gels populated with cells from older granulation tissue (28 days) and reduced the ability of cells from younger granulation tissue (7 and 14 days) to contract gels. Fibroblasts from different aged donors were evaluated for their ability to contract a collagen gel and showed no significant differences. The sensitivity to the stimulatory effects of TGF-ß were also examined which indicated that fibroblasts from donors of all ages increased their rate and extent of contraction following TGF-ß treatment to similar degrees (250).

A variety of studies have approached the problem of defining the contractile components of fibroblasts by contrasting normal cell strains to cell strains that are less capable of inducing contraction. Ehrlich and colleagues (80) compared normal human fibroblasts with recessive epidermolysis bullosa dystrophica (EBD) fibroblasts and found that the normal human fibroblasts had a faster contractile rate and significantly greater cellular elongation. They later attributed this lack of contraction of EBD fibroblasts to increased secretion of prostaglandin E_2 (PGE$_2$) (86). The production of PGE$_2$ could be normalized by treating the cells with indomethacin, a cyclooxygenase inhibitor, and a subsequent normal contraction rate

was observed. Ehrlich and Griswold (82) also examined the cAMP levels of these two cells lines and found that the elevated level of cAMP in EDB fibroblasts was normalized by indomethacin. The addition of cholera toxin, which stimulates adenylate cyclase, increased cAMP in normal cells to levels comparable to EDB fibroblasts. These two substances also affected the morphology and microfilament arrangement, converting them from the normal bipolar shape to a dendritic shape. This difference in morphology was also noted that when cells were permeabilized with glycerol and treated with ATP buffer which induced them to undergo shrinkage (83). The normal human fibroblasts underwent significant shrinkage of 72% which could be block by treatment with PGE_2, cholera toxin or cAMP. Steinberg and colleagues (298) compared normal and transformed human fibroblasts and found the transformed cells to be far less efficient at contracting the gels. Delvoye and colleagues (66) found no significant difference between normal human fibroblasts and fibroblasts from patients suffering from scleroderma or Ehler-Danlos Syndrome type I, but did find a significant reduction in the contractile ability of dermal fibroblasts from dermatosparactic calves compared to normal calf fibroblasts.

2.4 INTERFERON

2.41 Introduction

The term interferon (IFN) was originally coined following the observation that a "factor" was released from cells when challenged with a heat-inactivated influenza virus. This factor was found to "interfere" with viral infection, hence was referred to as interferon (156). Since that time the IFN cytokine family has grown to include a family of 16-25,000 dalton proteins which are classified according to their gene sequence. The interferons produced predominantly by leukocytes include IFN-α and IFN-ω. IFN-β is produced by fibroblasts and IFN-γ by immune cells. In addition to their antiviral effect, IFN's have been noted to possess antiproliferative and antifibrotic properties which has stimulated research exploring these properties, particularly in relation to fibroproliferative disorders. In order to capitalize on these properties for the treatment of fibrosis, a thorough understanding of the mechanism of action is required. This review will focus on IFN-α, with reference being made to IFN-β and IFN-γ for comparative purposes.

The intracellular steps that mediate the cellular responses associated with exposure to IFN, are initiated by and dependent upon, ligand binding to high affinity, species-specific cell-surface receptors. As depicted in Figure 1 the current model of the IFN signaling pathways begins with ligand binding to the respective multimeric receptors, followed by tyrosine kinase phosphorylation

of cytoplasmic DNA binding proteins that translocate to the nucleus and bind to specific cis-acting response elements that regulate transcription of a set of immediate-response genes.

2.42 Interferons

There have been 18 human IFN-α genes identified, four of which are pseudogenes (67, 166). The various IFN-α isoforms that are at least 70% homologous. They are clustered on chromosome 9 and do not contain any introns. Their products are 188 or 189 amino acids long with 23 amino acids being the hydrophobic signal peptide. As the protein is transported, the signal peptide is cleaved off resulting in a 165 or 166 amino acid mature protein. Unlike IFN-ß and IFN-γ, human IFN-α does not have any N-linked glycosylation sites, however evidence for O-linked glycosylation has been reported for several species of IFN-α (237). Two disulfide bonds are present in the mature IFN-α protein with cysteine residues 29 and 138 (139) in addition to 1 and 98 (99) being linked. IFN-ß has a single disulfide bond but IFN-γ does not have any (67). However the mature biologically active form is a homodimer whereas IFN-α and IFN-ß function as single polypeptides (94).

Comparisons of sequences suggest that IFN-α and IFN-ß were derived from a common ancestor by gene duplication. In addition, the lack of introns, which is an unusual gene structure feature that they have in common, supports this hypothesis. In contrast, the profound differences in gene structure as well as nucleotide and amino acid sequence between IFN-α/ß and IFN-γ suggests that they

are evolutionarily distinct. The fact that IFN-α/ß and IFN-γ interact with distinct high affinity receptors and display markedly different biologic activities, further supports their individual evolution (301). The common ancestry and overlapping induction of signal transduction pathways by IFN-α and IFN-ß has led to their designation as type I IFNs with IFN-γ being referred to as type II.

2.43 Receptors

The type I IFN receptor was initially identified as a specific and distinct receptor from the type II receptor on mouse (4) and human cells (28), using competitive inhibition of ^{125}I-labeled murine and human-αA interferon respectively. Subsequent attempts to identify, characterize and clone the receptor were complicated by initially contradictory reports. Recently an apparent consensus has been obtained that appears to reconcile the various reports (311).

A fair amount of confusion exists in the literature regarding the IFN-α receptor. Following gene transfer experiments, in which a cDNA for human IFN-α receptor was cloned and expressed in mouse cells rendering them sensitive to human IFN-αB (310), doubts arose as to whether this was an accessory subunit. The basis for this doubt was the fact that the mouse cells were only capable of binding human IFN-α8, although its presence was probably necessary for the activity of all IFN-α/ß isoforms. Further experiments subsequently proposed alternative possibilities, which have since been shown to represent same protein (43, 49, 317). Hence the original report has proven to be the ligand binding, transmembrane protein that is

responsible for the initiation of the type I IFN signal transduction cascade. An IFN receptor binding protein, which does not react with the IFN receptor antibody, has also been identified (44, 49, 87, 317). The 51 kD disulfide-linked homodimer, which was initially thought to represent the type I receptor (230), now appears to be either the same subunit identified above or a distinct subunit. The functional interaction of these various proteins have yet to be clearly delineated (311). Bovine and murine subunits have also been cloned which exhibit a significant degree of sequence homology and similar biologic function (217, 309) yet are likely responsible for the species specificity.

The type II receptor was initially purified from foreskin fibroblasts then characterized by crosslinking studies and antibody generation (231). This was soon followed by the isolation of the human IFN-γ receptor cDNA (5), which was found to encode a transmembrane protein (489 aa) of a predicted molecular weight of about 54 kDa with multiple potential N-linked and O-linked (extracellular domain) glycosylation sites (94). Analysis of wild-type and mutant receptor expression has determined that several functional domains exist. Proximal to the transmembrane region is a 48 amino acid residue sequence (256-303) that is required for receptor-mediated ligand internalization, containing within it a tetrapeptide (266-269), which is necessary for tyrosine phosphorylation and biological responsiveness (93, 123). Within the distal C-terminal region Tyr440, Asp441 and His444 were shown to be critical for IFN-γ biologic responsiveness (92). Tyr440 was both a target of tyrosine phosphorylation and critical for Stat91 (see

below) phosphorylation and signal transduction (123). Dimerization of the receptor follows binding of IFN-γ (94, 124).

Using the human cDNA to probe murine libraries the cDNA for the murine receptor was isolated and found to be approximately 52% homologous to the human receptor (121, 144). Transfection experiments conducted following the isolation of the IFN-γ receptor (α-chain) cDNAs, indicated that although the receptors were competent to bind ligands, cofactors were required to confer biologic responsiveness (148, 164). Recent work in human (295) and murine (143) cells has identified the species-specific accessory proteins (β-chain) and isolated the cDNA. Interestingly there is evidence that the α-component of the receptor is not species-specific, since studies where a mouse/human hybrid α-chain was transfected into a murine cell line lacking the murine α-chain successfully reconstituted signal transduction (26).

2.44 Tyrosine Kinases

The initial experimental evidence that protein kinase activity mediates the induction of the IFN-α transcriptional response was that IFN-stimulated gene factor-3α (ISGF3α) activation (see below) was inhibited when cells were treated with protein kinase inhibitors (62, 169, 251). Identification of the protein kinases involved in phosphorylation has been facilitated by genetic complementation experiments using mutant cell lines defective in various components of the signal transduction pathway (Table III). The phenotypic unresponsiveness of mutant human cell line U1A to

IFN-α was corrected following transfection of a wild-type gene (323). The gene was identified as Tyk2 following cross-hybridization with cDNA libraries from Daudi cells. Tyk2 had previously been described as a non-receptor protein tyrosine kinase with the tyrosine kinase motifs near its C-terminus (98). It is expressed as a single-copy gene encoding a putative polypeptide of 1187 aa (134 kDa) which is believed to link the interferon α/β receptor and the cytoplasmic transcription factors. The fact that Tyk2 was known to belong to a family tyrosine kinases, referred to as Janus or Jak proteins, whose function had previously been undefined (136) led to suggestions that the other Jak family members may also be involved in the IFN phosphorylation cascades. Subsequent experiments using a cell line that lacks Jak1 (mutant U4A) revealed that transfection with Jak1 cDNA expression constructs restored responsiveness to IFN-α/ß and IFN-γ (218). Restoration of the responsiveness to IFN-γ of the mutant cell line γ1A was achieved by transfection with a Jak2 expression construct (329). This defined an interdependence of action between Jak1 and Tyk2 in response to IFN-α treatment and between Jak1 and Jak2 in response to IFN-γ.

The role of the Jak tyrosine kinase family has been further supported. Jak1 phosphorylation was increased in mouse 3T3 cells treated with IFN-α or γ and Jak2 phosphorylation in response to IFN-γ. Further, kinetic analysis of the phosphorylation of Jak1 and Stat91 reportedly correlated with previous experiments showing a more rapid response to IFN-α. The overexpression of either Jak1 or Jak2 resulted in activation of Stat91 DNA-binding activity and IFN-γ

independent transcription (287). IFN-γ receptor immunoprecipitated with Jak1 prior to IFN-γ treatment in an unphosphorylated conformation and with both Jak1 and Jak2 in a phosphorylated state following IFN-γ treatment, indicated post-ligand binding recruitment of Jak2 (152). It has also been shown that IFN-α induces tyrosine phosphorylation of Tyk2 and was coprecipitated with the α-subunit of the receptor (45).

2.45 Cytoplasmic Proteins

The cytoplasmic substrate proteins involved in transcriptional activation differ for type I and II IFNs yet overlap. To date the most thoroughly defined factors is the protein complex, IFN-stimulated gene factor-3 (ISGF-3), which pre-exists in the cytoplasm and following IFN-α treatment is activated, then translocated to the nucleus (58, 170, 192). The ISGF-3 complex consists of two subunits, ISGF3α and ISGF3γ. Gel-shift analysis demonstrated that ISGF3γ was a 48 kDa protein and ISGF3α comprised three polypeptides (84, 91 and 113 kDa) (171), now referred to as signal transducer and activator of transcription 48 (Stat48), Stat84, Stat91 and Stat113 respectively (Table IV). In addition ISGF3γ was shown to have intrinsically low-affinity DNA-binding activity. Conversely ISGF3α's polypeptides did not have DNA-binding activity but when associated with ISGF3γ exhibited a 25-fold higher affinity than ISGF3γ alone (103, 171). Deletion analysis localized the DNA binding region of ISGF3γ to amino acids 10-117 and the region that interacts with ISGF3α to amino acids 217-377 (321). In addition,

mapping of the ISGF3-binding site suggested that ISGF3α specifically recognizes the 3' end of the interferon stimulated response element (ISRE, see below).

Purification and cloning of the cDNAs encoding the Stat proteins (104, 270, 322) has enhanced the understanding of the overlapping roles that these proteins play in signal transduction. The divergence of the cytoplasmic protein activation pathways became apparent when it was demonstrated that phosphorylation, which was essential for DNA-binding, occurred only on Stat91 of cells treated with IFN-γ (285) whereas treatment with IFN-α resulted in phosphorylation of Stat84, Stat91 and Stat113 (134, 271). Sequence comparison identified conserved Src homology (SH) 2 and SH3 domains in Stat84, Stat91 and Stat113 which are thought to play an important role in interactions between tyrosine kinases and tyrosine phosphorylation regulated effector proteins (102). Further analysis of the Stat84 and Stat91 proteins established that they were encoded by a single gene with Stat84 lacking 38 amino acids at the carboxy-terminus as a result of alternative splicing (270). The residue which is phosphorylated following IFN-γ treatment was shown to be Tyr[701] on both Stat84 and Stat91 (286). Translocation of Stat84 to the nucleus did not result in transcriptional activation (219). It has more recently been reported that Stat91 exists as a monomer prior to IFN-γ ligand-binding but following phosphorylation forms a homodimer which is mediated through SH2 phosphotyrosyl peptide interaction (285). Conversely it has been demonstrated that following stimulation with IFN-α, phosphorylation of Stat91 or Stat84 on Tyr[701] is required for ISGF-3

formation and efficient nuclear translocation and that the phosphorylation of Stat113 on Tyr^{690} is independent of Stat84 or 91 (155).

Following the cloning of Stat91(84) and Stat113 it became apparent that there is extensive cross-talk between the signal transduction pathways of the IFNs and other growth factors (31, 154, 166) which raises many questions regarding interaction between pathways following ligand binding. Some of these question have been answered following the production of a Stat91 deficient mouse which displayed unresponsiveness to either IFN-α or -γ yet maintained normal responsiveness to several cytokines that activate Stat1 *in vitro* (213).

A second set of transcriptional regulators have been described and are referred to as the interferon regulatory family (IRF). IRF-1 and IRF-2 have been cloned and shown to bind to the same DNA-binding site as the Stat proteins with IRF-1 acting as a transcriptional promoter and IRF-2 competing for binding and acting as a suppressor protein (135, 214, 222, 240). A third member of the family, interferon consensus sequence binding protein (ICSBP), was isolated from a cDNA library (72). Mobility shift experiments have demonstrated that ICSBP has an increased affinity for DNA binding when a complex is formed with IRF-1 or IRF-2 and in turn competes for binding with Stat48 (27). Unlike Stat48 and IRF-1, ICSBP is an inhibitor of interferon stimulated gene expression (222). Sequence homology analysis has revealed that towards the amino terminus of Stat48 protein, where DNA-binding was shown to occur (321), there is significant sequence homology with the IRF family of proteins,

ICSBP and *myb* oncogene products, suggesting the use of a common structural motif for DNA recognition.

Several other trans-acting factors have been identified which regulate the expression of interferon itself as well as the previously recognized interferon-inducible genes (see below) but their function in humans and their physiologically relevant inducers have not been well defined (166).

2.46 Inducible Genes

Although the induction of gene transcription is the final step of the signal transduction pathway, the actual inception of the search to define the steps involved, began when several groups demonstrated that there was a set of specific target genes that were rapidly transcribed following activation by interferons (interferon-stimulated genes, ISGs) (101, 168, 185, 335). The 5' flanking region of the ISGs was found to contain a response element (ISRE). This region was defined experimentally by fusion experiments that conferred IFN responsiveness when the ISRE was fused upstream to a previously unresponsive gene or a marker gene (193, 194, 245, 252). Mutation experiments further defined the essential regions of this cis element which has a conserved 15 bp region with a core 9 bp element which abolishes IFN responsiveness if mutations occur within it (59, 193, 245, 252). This region however has not met the requirements for the induction of genes by IFN-γ. Examination of early response genes following IFN-γ treatment has resulted in the description of several elements required for

transcription. The similarity between these promoter elements has led to the proposal of an IFN-γ-activated site (GAS) (166).

As depicted in Figure 1 recent progress has provided a fairly complete picture of the signal transduction pathway which is activated following IFN ligand-binding. However many questions remain unanswered. Although the receptors for type I and type II IFN have been identified and cloned, the localization and the nature of the association between the accessory cofactors has yet to be fully defined. Assignment of the functionally important Asp441 and His444 residues of the IFN-γ receptor (92) has yet to be completed and may play a key role in the interaction of accessory factors. Information regarding the role of the Jak kinase family of proteins is being produced at a rapid rate yet questions remain. One of the most exciting challenges being conclusive demonstration of the sequence of events in the functional link between the respective receptors, Jak kinases and Stat proteins. Since Jak1 and Jak2 could not be coprecipitated with Stat91 (123) it is possible that another, unidentified effector protein is involved.

2.47 Antifibrogenic Effect

The suggestion that interferons may function as anti-fibrogenic factors has led to *in vitro* investigations of its effects in several fibroproliferative disorders. Fibroblast growth and collagen production were inhibited in normal fibroblasts (160) and scleroderma fibroblasts exposed to interferons (75, 165). Hypertrophic scar and normal paired fibroblasts treated with IFN-γ

were shown to have a reduced rate of proliferation, collagen production and mRNA expression of type I and type III procollagen (139). When treated with IFN-α2b, a reduction in the rate of fibroblast proliferation and fibronectin, type I and type III collagen mRNA was observed (114, 306). Interestingly the treatment effect was delayed, which suggested the effect may be secondary to new protein synthesis. Down-regulation of nuclear factor-1, which is a procollagen gene-activating transcription factor may account for the time lag (76).

The development of contractures in post-burn HSc may also be reduced by interferon treatment. Using the fibroblast-populated collagen lattice IFN-α2b, IFN-ß and IFN-γ have been shown to reduce the rate and extent of lattice contraction (60, 221). Similiar to the experiments outlined above there was a treatment-time dependent reduction in contraction (221) which again suggests the requirement for new protein synthesis rather than a direct effect. Further, mRNAs for actin isoforms ß and γ were significantly reduced following IFN-α2b treatment. Pfeffer and colleagues (238, 239) examined morphologic changes in human fibroblasts when treated with interferons in culture. These included 1) inhibition of redistribution of cell surface components, 2) enlargement of cells, 3) presence of large stress fibers, 4) a long fibrous array of fibronectin on the cell surface and 5) a decrease in locomotion and intracellular movements. Morphologically the interferon-treated fibroblasts within the gel exhibited a more spread out arrangement of stress fibres (221), similiar to that seen on plastic. These findings suggest that treatment with interferons may counteract the

increased proliferation, excessive extracellular matrix production and increased contraction that is induced by fibrogenic growth factors, thereby facilitating the resolution of HSc.

The data obtained from *in vitro* studies has supported the initial trials of interferons *in vivo*. In a murine skin wounding model Grainstein and colleagues (119) demonstrated that systemic administration of IFN-γ inhibited collagen synthesis and the acute inflammatory response. Intralesional injection of IFN-α2b (24) or of IFN-γ (120) led to a reduction in keloid mass in humans. Similarly beneficial effects were noted following intralesional injections of IFN-γ for the treatment of HSc and Dupytren's disease (242).

2.5 Thesis Aims and Experimental Rationale

At the present time the treatment of scar contractures is unsatisfactory. It is our hypothesis that the therapeutic use of interferon for the treatment of fibroproliferative disorders may have the additional benefit of inhibiting scar contraction. In order to investigate this hypothesis the effect of IFN-α2b in an *in vitro* model system which simulates wound contraction was evaluated. Since it was anticipated that any effect that IFN-α2b had would be mediated through alterations in cytoskeletal protein expression, specific quantitative and semi-quantitative techniques were utilized to assess the mechanism by which it has its effect.

The specific aims of the study were to:

I. evaluate the ability of HSc and normal patient-matched fibroblasts to induce contraction in the fibroblast-populated collagen lattice model system.

II. assess the effect that exposure to IFN-α2b and fetal bovine serum has on the rate and extent of contraction.

III. quantitate the IFN-α2b induced changes in the steady state mRNA levels of cytoskeletal proteins which are likely to be important in the contractile process.

IV. visualize alterations in the morphology of fibroblasts within the lattice as a function of time and IFN-α2 b treatment.

Generalization of research findings from an *in vitro* model system to the human condition is often associated with erroneous assumptions and experimental artifacts, however the inhibition of contraction in the *in vitro* model system supported more elaborate investigation within an *in vivo* animal model system. This series of investigations expanded our understanding of the effect of IFN-α2b on wound contraction within a multifaceted biological system.

The specific aims of the study were to:

I.   evaluate the effect of systemic administration of IFN-α2b on wound contraction in an *in vivo* animal model.

II. quantitate the expression of cytoskeletal proteins that are likely to be important in the contractile process and the effect of IFN-α2b on programmed cell death.

III. examine the *in situ* expression of myofibroblast-specific cytoskeletal protein α-smooth muscle actin and decorin as a function of time post-wounding and establish the effect that IFN-α2b treatment has on its expression.

Experimental manipulation of human scar contraction is ethically and technically difficult, however it is important to correlate the *in vitro* and *in vivo* animal model findings to that of the human counterpart, particularly when scar formation differs so substantially from that observed in animals.

The specific aims of the study were to:

I. evaluate the presence of myofibroblasts in a spectrum of human postburn scars including burn scar that developed HSc and burn scar that developed normal scar, patient-matched partial thickness wounds that developed normal scar and HSc from patients treated with systemic IFN-α2b.

II. correlate the presence of myofibroblasts, total number of fibroblast and percentage of apoptotic cells with pathologic wound healing in humans and evaluate changes associated with resolution and systemic administration of IFN-α2b.

The information obtained from this research will provide us with new insights regarding the mechanisms involved in wound contraction and scar contracture formation, that will in turn enhance our understanding of the potential benefit of IFN-α2b in the treatment of burn survivors.

2.6 Figures

Table 1 Cytokines released at the injury site. Following wounding various cell types are recruited into the wound and are responsible for the production and release of cytokines that in turn act as chemoattractants, mitogens, and stimulants of protein production.

Cell Type	Time in Wound	Cytokine Produced	Reference
Platelets	0-6 hours	TGF-ß	13
		TGF-α	150
		PDGF	256, 178
		TNF	246
		IL-1	246
		EGF	234
		IGF-1	167
		bFGF	
Neutrophils	1st-2nd day	TGF-ß	56, 129
Macrophages	2nd-14th day	TGF-ß	13
		TGF-α	249
		PDGF	283
		IL-1	150
		bFGF	16
		MDGF	190
		TNF-α	189
		IFN-ß	99
		IFN-α	176
		IFN-γ	
Epithelial	6 hours-3rd day	PDGF	9
Mast Cells		EDF	88
		TNF-α	
Endothelial	3rd-14th day	PDGF	70
Fibroblasts	2nd day onward	IFN-α	

Table 2   The expression of α-smooth muscle actin in fibroblasts has been associated with a wide range of pathologies.

PATHOLOGY	REPRESENTATIVE REFERENCES
Bladder Obstruction	32
Cardiac Disorders	331, 334
Dupytren's Disease	293
Granulation Tissue	61
Hypertrophic Scar	81
Liver Disease	89, 229, 247, 273, 281, 340
Ophthalmologic Disease	149, 274, 327
Oral Pathology	198
Pulmonary Fibrosis	183, 324, 343
Renal Disease	147, 161, 204
Scleroderma	266
Tumors	57, 140, 162, 177, 191, 195, 276, 277, 291, 293, 307, 308
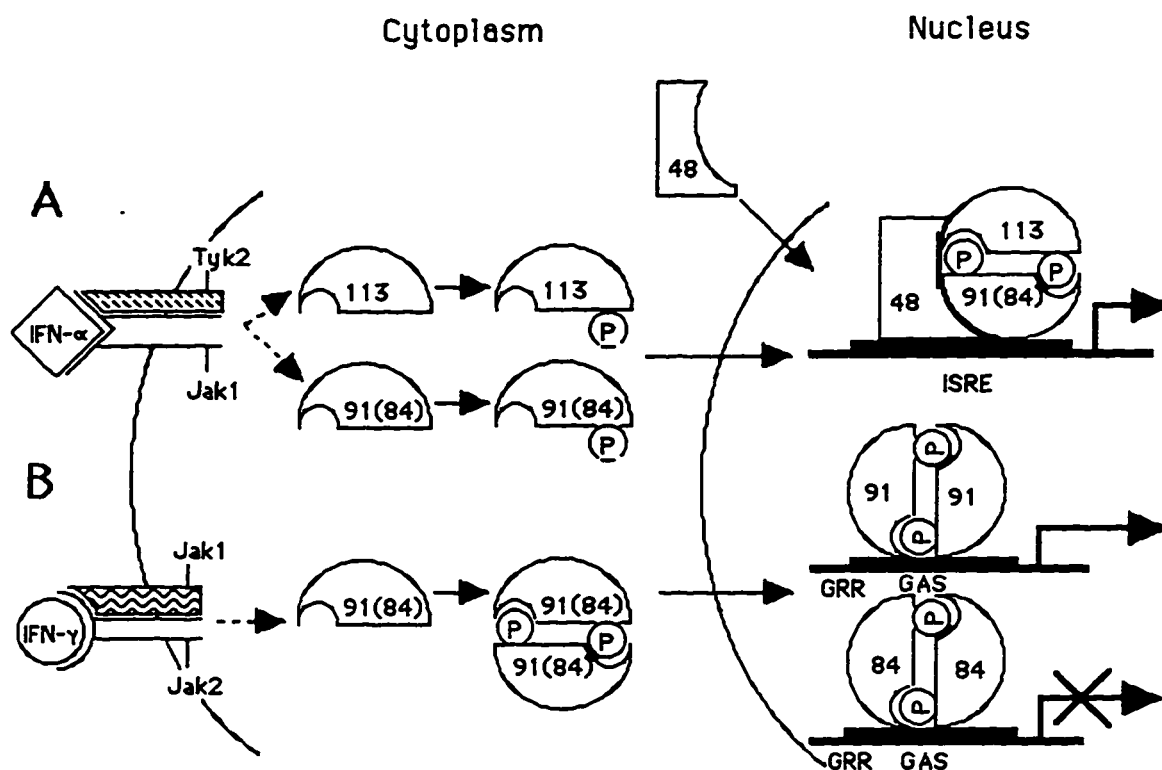
Figure 1   Interferon signal transduction pathway.

A: Model for type I interferon signal transduction. Following ligand binding to high-affinity receptor and cofactor, latent Stat proteins 84, 91 and 113 are rapidly activated through phosphorylation by non-receptor protein-tyrosine kinases Tyk2 and Jak1. These proteins form a complex, migrate to the nucleus, combine with Stat48 and following DNA-binding at the ISRE activate transcription. Other less well defined factors may also bind to ISRE (refer to text).

B: Model for type II interferon signal transduction. Following ligand binding to high-affinity receptor and cofactor, latent Stat proteins 84 and 91 are rapidly activated through phosphorylation by non-receptor protein-tyrosine kinases Jak1 and Jak2. These proteins form a dimer, migrate to the nucleus and following DNA-binding at GAS Stat91 dimer activates transcription but Stat84 dimer does not. GRR is an additional element required for transcriptional activation.

Table 3 Mutant Cell Lines. Mutant cell lines that are lacking components of the interferon signal transduction pathway have been used to identify and clarify the steps involved.

Name Reference		Defect
γ1A	(38)	defective Jak2 production
U1A=11.1	(34)	lacks protein tyrosine kinase Tyk2
U2A	(87)	truncated and inactive Stat48
U3	(56)	lacks Stat91 and 84 mRNA
U4A	(37)	lacks Jak1

Table 4 Nomenclature of cytoplasmic proteins: As the components of the interferon signal transduction pathway have been identified and better understood the names have changed to better reflect their roles and interrelationships.

p48	(ref 45)	=	Stat48	(ref 55) =	ISGF3γ	(ref 45)
p84	(ref 45)	=	Stat84	(ref 55) =	Stat1β	(ref 87)
p91	(ref 45)	=	Stat91	(ref 55) =	Stat1α	(ref 87)
p113	(ref 45)	=	Stat113	(ref 55) =	Stat 2	(ref 87)
p113 + p91 or						
p113 + p84	(ref 45)	=	ISGF3α	(ref 45) =	E factor	(ref 43)

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CHAPTER 3:

THE EFFECT OF INTERFERON ALPHA-2B ON THE EXPRESSION OF

CYTOSKELETAL PROTEINS IN AN IN VITRO MODEL OF WOUND

CONTRACTION

3.1 ABSTRACT

Wound contraction is an essential component of wound healing, however the development of scar contractures in tissues and organs disrupts normal organ integrity and produces functional deformities. Although interferons alpha and gamma inhibit extracellular matrix protein production by fibroblasts, their effects on cytoskeletal protein mediated-wound contraction are as yet unclear. The fibroblast-populated collagen lattice is an in vitro assay which simulates wound contraction. Using matched pairs of human hypertrophic scar and normal dermal fibroblast cultures established from patients recovering from a thermal injury, interferon-α2b exposure prior to lattice formation was found to significantly inhibit contraction in a treatment time-dependent manner ($p < 0.05$). Fibroblasts generated contractile forces that were triphasic and serum sensitive ($p < 0.01$). Comparison of hypertrophic scar and normal dermal fibroblasts revealed no significant differences in ability to induce lattice contraction. Northern blot analysis of mRNAs for the intracellular contractile proteins revealed that interferon-α2b significantly down-regulated mRNA levels of the actin isoforms β and γ (50-60%) but had no significant effect on α-tubulin, vimentin and α-actinin. Fibroblast-populated collagen lattices were stained with rhodamine-labeled phalloidin to reveal filamentous actin proteins. Marked morphologic alterations of the stress fibres were associated with reductions in lattice contraction following interferon-α2b treatment. Thus, interferon-α2b's inhibition of wound contraction in vitro is associated with

reductions in mRNA for β and γ actin and distinct morphologic alterations in fibroblast stress fibre morphology.

3.2 INTRODUCTION

Contraction, as a normal feature of wound healing, is an important element in cutaneous repair following tissue injury to the skin (39). However, pathologic or excessive wound contraction is a major factor contributing to the loss of function and re-establishment of organ integrity in many fibroproliferative disorders, including hepatic cirrhosis (43), scleroderma (33), Dupuytren's disease (44), hypertrophic scar (HSc) (34) and pulmonary fibrosis (42).

Although the cellular and molecular mechanisms have yet to be fully explained, it has been determined that wound contraction is an active fibroblast-based process (3). The fibroblast-populated collagen lattice (FPCL) provides an in vitro system in which the interaction of dermal fibroblasts with a collagen matrix can be examined and the effects of potential treatments quantified. As one phase of wound healing, the process of contraction is almost certainly regulated by various growth factors and chemoattractants (29). During normal wound healing in the skin, an orderly fibrotic response, initiated by the infiltration of activated fibroblasts into the damaged region, concludes in the formation of scar tissue. With fibrotic conditions of the dermis such as HSc or keloids significant increases in the expression of extracellular matrix components such as glycosaminoglycans (29, 39), collagen type I and collagen type III

(43, 21) occur. It has been suggested that these characteristics may be attributed to the fibrogenic effect of growth factors such as transforming growth factor-ß (TGF-ß) (49, 52). In vitro, interferon-alpha 2b (IFN-α2b) reduces collagen synthesis (50) and fibronectin mRNA expression (35) in human HSc fibroblasts and normal dermal fibroblasts. In addition, interferon-gamma (IFN-γ) treatment decreased collagen production in vivo in a murine wound healing model (51). Trials of intralesional injections of IFN-γ (36) and IFN-α2b (44) have provided evidence that scar maturation is accelerated following treatment. The suppression of contraction in vitro by IFN-α2b has been demonstrated in HSc, keloid and unmatched normal dermal fibroblasts (44) as well as human neonatal fibroblasts (11), but further investigation of the components of the contractile process that are potentially affected by interferon treatment is required.

The contractile properties of HSc and normal fibroblasts were investigated using paired fibroblast strains obtained from the same patient to control for age, sex, and other factors. Using the FPCL assay, the stimulatory effects of serum and the inhibitory effects of IFN-α2b were investigated. To understand the mechanism by which lattice contraction was inhibited, the effect of IFN-α2b on the levels of mRNA for a number of cytoskeletal proteins during wound contraction in vitro was examined. Active contraction of fibroblasts in the FPCL model was correleated with the level of mRNA for the cytoskeletal proteins actin, tubulin, actin-associated and intermediate filament proteins. Finally, histologic analysis of IFN treated and control fibroblasts within the collagen gels, as well

as the appearance of filamentous actin within the FPCL stained with rhodamine-labeled phalloidin, was conducted to correlate the morphology and appearance of stress fibres and actin microfilaments in HSc and normal fibroblasts with the degree of contraction observed in vitro.

3.3 MATERIALS AND METHODS

Clinical Specimens

As previously described (50) paired dermal biopsy specimens were obtained from five patients who developed HSc following a thermal injury who were cared for at the University of Alberta Hospitals Firefighters' Burn Treatment Unit (Table 1).

Cell Cultures

Strains of HSc and normal dermal fibroblast cultures were established as described by Nakano and Scott (35). Briefly, punch biopsies were obtained from hypertrophic scar and normal skin of post-burn injury patients following informed consent. The tissue was collected in DMEM with 10% FBS (Gibco, Grand Island, NY), minced into small pieces of less than 0.5 mm in any dimension, washed six times with sterile medium and distributed into 60 x 15 mm Petri culture dishes (Falcon, Oxnard, CA), four pieces per dish. A sterile glass coverslip was attached to the dish with a drop of sterile silicone grease to immobilize the tissue fragments. DMEM +

Ab (penicillin G sodium 100 U/ml, streptomycin sulfate 100 μg/ml and amphotericin B 0.25 μg/ml) (3 ml) with 10% FBS was added to each dish and incubated at 37°C in a water jacketed humidified incubator in an atmosphere of 5% CO_2. The medium was replaced twice weekly. After 4 weeks of these conditions the cells were released from the dishes by brief (5 min) treatment with 0.1% trypsin and 0.02% EDTA (Sigma, St. Louis, MO) in PBS (pH 7.4) and transferred to 75 cm_2 culture flasks (Corning Inc., NY, NY). Thereafter, once visual confluence was reached the cells were subcultured 1:6 by trypsinization. Cell cultures from similar passage number (three to seven) were used for the fibroblast-populated collagen lattice assay, RNA extraction and rhodamine-labeled phalloidin staining.

Interferon Alpha-2b Treatment

Fibroblasts were grown in 75 cm_2 culture flasks in 10 ml of DMEM/10% FBS, which was changed every three days. Once visual confluence was reached, HSc and normal fibroblasts were exposed to IFN-α2b (Schering Corporation, Bloomfield, NJ; specific activity 2 x 108 IU/mg protein) at 2000 IU/ml, which maximally reduced type I collagen mRNA as previously described (50). Fibroblasts were continuously exposed to IFN-α2b for 144, 96, 48 or 0 h prior to harvesting for the FPCL assay. Fresh medium was added every three days with all treatment groups being harvested at the same time to control for duration of time at confluence. In the first experiment one group was treated only at the time of collagen gel

polymerization to establish whether there was a direct effect of IFN treatment. This control group was omitted in subsequent experiments as no difference was seen relative to the untreated control (see below). Once polymerized within the collagen lattice, the fibroblasts received continuous exposure to IFN-α2b at 2000 IU/ml with the exception of the control. For experiments using 5% FBS, fibroblasts were maintained at 10% FBS up to the time of incorporation into the FPCL and then the FBS concentration was decreased to 5%. For northern blot analyses and rhodamine-labeled phalloidin experiments, HSc and normal fibroblasts were exposed to IFN-α2b for 144 h at which time maximal inhibition of contraction was observed (see below).

Fibroblast-Populated Collagen Lattice Assay

Fibroblast-populated collagen lattices were made using bovine type I collagen extracted as described by Volpin and Veis (51) using a modification of the procedure of Bell et al (33). The following experiments were undertaken with four different paired cell strains at 10% FBS concentration and one paired cell strain at 5% FBS concentration. The lattices were produced in 35 mm bacterial petri dishes (Falcon, Oxnard, CA). Each dish contained 350 μl 3x DMEM + Ab, 26 μl 0.4 M NaOH, 440 μl cell suspension (1 x 106 cells/ml) in 1x DMEM + 10% FBS, 125 μl FBS (for a final concentration of 10%) or 40 ml FBS (for a final concentration of 5%) and 870 μl acid-extracted fetal bovine type I collagen (2.14 mg/ml). These conditions produced rates of contraction in the control groups

similar to those reported previously (33, 36, 34). 2000 IU/ml of IFN-a2b was added to the gels at the time of polymerization where appropriate. Each treatment group was prepared in triplicate and immediately transferred to a humidified incubator at 37°C in an atmosphere of 5% CO_2 .

The surface area of the collagen gels was measured after 2, 4, 6, 8, 10, 12, 24, 48 and 72 h using a transparency overlay with a dark background for contrast. The images were quantified on a HIPAD™ digitizing tablet (model DT11AA, Houston Instrument) connected to an IBM-PC computer equipped with a planimetry program (Sigma Scan™ 2.5, Jandel Corporation, Sausalito, CA) to determine the integral of free-form diagrams. The data are expressed as percentages of the original surface areas.

Cell viability within the collagen lattice was determined after 72 hrs for all fibroblast-populated collagen lattice experiments. Cells were released by digesting the lattice with bacterial collagenase (Sigma, St. Louis, MO) in PBS (4 mg/ml) at 37°C for 30 min. Isolated cells were pelleted by centrifugation at 1600 rpm for 8 min and resuspended in 0.04% trypan blue (Sigma, St. Louis, MO) in PBS. The cells were mounted on a hemacytometer and viability determined by the percentage of cells that excluded the dye.

Northern Blot Analysis

Four paired strains of HSc and normal fibroblasts were grown to confluence and treated with IFN-α2b as described above. Total RNA was extracted from IFN-α2b-treated and untreated HSc and

normal fibroblasts and hybridized with specific cDNA probes as previously described (21). Briefly, the cells were washed twice with 10 ml of ice-cold PBS, then lysed and pooled in 6 ml of guanidinium thiocyanate (GITC). The cell lysate was transferred to 50 ml centrifuge tubes and the DNA was sheared using a 20 ml syringe with an 18 gauge needle. The total RNA from each individual cell lysate was then extracted using the GITC/CsCl procedure of Chirgwin et al (29) and separated by electrophoresis on a 1% agarose gel containing ethidium bromide and 2.2 M formaldehyde. Gels were then blotted onto nitrocellulose membranes (48). The membranes were baked in a vacuum oven for 2 h at 80°C, then incubated in pre-hybridization solution for 2-4 h at 42°C (21). Hybridization was performed at 42°C in the same solution for 16-20 h using cDNA probes for ß or γ-actin, α-actinin, α-tubulin, or vimentin (American Type Culture Collection, MD). The cDNA probes were labeled with ^{32}P-alpha-dCTP by nick translation. The membranes were washed at room temperature with 2x SSC and 0.1% SDS for 1 h, then for 30 min at 65°C in 0.2 x SSC and 0.1% SDS. Autoradiography was performed by exposing Kodak X-Omat film to the nitrocellulose membranes at -80°C in the presence of an enhancing screen. Autoradiograms were later quantified by densitometry.

Confocal Microscopy of Collagen Lattices

Rhodamine-labeled phalloidin staining of HSc and normal dermal fibroblasts in the FPCL was performed after 2 h or 24 h on the gels fixed in 4% paraformaldehyde for 5 min then extensively

washed with PBS (pH 7.4). Tetramethylrhodaminyl isothiocyanate-phalloidin (TRITC-phalloidin) (Sigma, St. Louis, MO) was diluted to 2.5 µg/ml in ethanol, applied to the gels and incubated at room temperature for 30 min. Cells were examined by confocal laser scanning microscopy (Leica Lasertechnik, Heidelberg) using a standard fluorescence Leitz Aristoplan microscope equipped with an argon/krypton laser. Optical excitation was with the 514 µm line of the laser. The emitted fluorescent light passed through a 530 µm (OG 530) long-pass filter before it reached the photomultiplier. Horizontal scanning was done by a moving laser beam and optical sectioning along the z-axis was achieved by a high precision focusing stage. Images were averaged over 32 scans in the line scanning mode. Slides were produced on a slide processor (Agfa Corporation, Wilmington, MA) from the NIH Image program following shadowing enhancement to facilitate visualization.

Statistical Analysis

Data on the extent and rate of contraction in the FPCL assay were analyzed by multiple analysis of variance (MANOVA) using the SPSS-X statistical package (SPSS Statistical Program, Chicago). Data from the northern analysis for IFN-α2b treated cells were compared with those of the untreated controls using Student's paired t test. P values of <0.05 were considered significant.

3.4 RESULTS

Fibroblast-Populated Collagen Lattice

The combined data from four cell strains at 10% FBS revealed that the treatment groups underwent a three-phase pattern of gel contraction with a short attachment or "lag" phase, followed by a 24 h period of rapid reduction in surface area which tapered off thereafter, until contraction was complete at 72 hrs (Figure 1). Control fibroblasts not exposed to IFN-α2b showed the greatest reduction in gel surface area: hypertrophic 79±2%; normal 79±1% (% reduction±SEM). As the duration of exposure to IFN-α2b increased, the contractile abilities of HSc fibroblasts were reduced to 72±3%, 66±3% and 57±4% after 48 h, 96 h, and 144 h respectively (Figure 1A). Similarly, the contractile abilities of IFN-α2b-treated normal fibroblasts were reduced to 72±3%, 69±5%, and 65±3% (Figure 1B). IFN induced reduction in contraction was statistically significant in all treatment groups (n=12 for each group: MANOVA $p<0.05$). Statistical comparison of HSc fibroblasts and normal dermal fibroblasts from the same patient revealed that the origin of the cell strain, when comparing the identical treatment conditions, did not significantly affect the rate or extent of contraction (data not shown). Fibroblasts which were not exposed to interferon before being placed in the lattice but were treated at the time of lattice polymerization and until contraction was complete, did not demonstrate reduced contraction or any cytotoxic effects (data not

shown). Therefore, IFN-α2b did not appear to have a direct effect on the ability of the cells to contract the lattice.

Lattice contraction was significantly reduced by the addition of 5% FBS as compared to 10% in the HSc and normal fibroblasts (Figure 2). Treatment with IFN-α2b inhibited lattice contraction in all 5% FBS treated cells, with the 144 h treatment groups demonstrating a complete lack of contraction during the entire 72 h evaluation period (Figure 2B and 2D). As was the case with the fibroblast-populated lattice at 10% FBS, fibroblasts which were not exposed to interferon before being placed in the lattice but were treated at the time of lattice polymerization and until contraction was complete, did not demonstrate reduced contraction or demonstrate any cytotoxic effects as determined by cell viability (data not shown).

Expression of mRNA for Cytoskeletal Proteins

Northern blot analysis of total RNA extracted from two paired cell strains of 48, 96, 144 h IFN-a2b treated and untreated cells was carried out. The message levels that were effected by interferon treatment showed a gradual reduction with the maximum effect at 144 h (data not shown) therefore 0 and 144h treatment was used for the remaining two experiments (see below). Ethidium bromide staining of 28S and 18S ribosomal RNA were used as an initial loading control (Figure 3 and 4, Panel C). Hybridization of the cDNA specific for α-actinin indicated that the expression of this transcript did not vary with IFN-α2b treatment and therefore it was subsequently used as an additional internal loading control (Figure

3-6, Panel B). The autoradiograms for the other cytoskeletal proteins were quantified by densitometry and their relative expression adjusted for loading using α-actinin.

The γ and β actin isoform mRNAs had apparent sizes of 2.1 kb and were less abundant after treatment with IFN-α2b (Figure 3A and 4A). IFN-α2b treatment significantly reduced γ-actin mRNA in the HSc cell strains (to 38±3% of control) (Figure 3D), and β-actin mRNA in the normal cell strains (49±12%) (Figure 4D) ($p<0.05$). The reduction in γ-actin mRNA in the normal cell strains (37±5%) (Figure 3D), and β-actin mRNA in the HSc cell strains (49±6%) (Figure 4D) were marginally significant ($p<0.06$). The 1.9 kb transcript for α-tubulin was less abundant after treatment with IFN-α2b (HSc, 79±25%; normal, 68±15%) but these changes were not statistically significant (Figure 5). Conversely, the expression of vimentin was not affected by exposure to IFN-α2b (HSc 106±9%; normal 102±22%) (Figure 6).

Filamentous Actin

Examination of the filamentous actin 2 h after polymerization of the gels revealed similar morphology and orientation for both IFN-α2b-treated and untreated cells (Figure 7A and B). Actin filaments were seen to extend three dimensionally in a star-like or arborized pattern and with comparable intensity of staining in each of three dimensions of their projecting limbs. At this time, surface area measurements of the FPCL demonstrated no detectable differences between IFN-α2b-treated and untreated cells. However,

after 24 h within the gel marked variations in the organization of the filamentous actin were seen (Figure 7C and D). Untreated fibroblasts became flattened and oriented in a bipolar-spindle or elongated shape as the stress fibres consolidated towards the apices of the cell (Figure 7C). IFN-α2b-treated fibroblasts were less elongated and lacked the bipolarity and unidirectional orientation (Figure 7D). The stress fibres appeared to be spread throughout the cytoplasm in a sheet, with focal adhesions that fanned outward as compared to the more focused, apical terminations seen in the untreated cells where actin was concentrated close to the cell membranes. These observations were made at a time when the surface area of the FPCL was reduced by approximately 65%. Fibroblasts derived from the HSc and normal dermal tissue did not differ significantly from one another in appearance when examined by this technique (data not shown).

3.5 DISCUSSION

Contraction of fibroblast-populated collagen lattices occurs in three phases: a lag phase, a rapid contraction phase and a slow contraction phase (36). Treatment of HSc and normal fibroblasts with IFN-α2b did not affect the lag phase but decreased the rate and extent of the contractile phases, thus suggesting that interferon treatment effects were on the mechanism of contraction rather than on cell attachment.

Similar to other investigations (34, 29) this study demonstrated that the rate of contraction was sensitive to serum

concentration, which is probably due to the presence of growth factors such as TGF-ß (7) and PDGF (30) in serum. Many cytokines have been shown to stimulate lattice contraction in the collagen gel model, including TGF-ß (51, 34), PDGF (10, 25), bFGF and TGF-α (51). In contrast, treatment of HSc and normal fibroblasts with IFN-α2b prior to incorporation within the gel markedly reduced the rate and extent of contraction in a time-dependent manner. This IFN-α2b-induced reduction in contraction was not the result of cytotoxicity because contraction was not inhibited by exposure to IFN-α2b at the time of lattice polymerization but required 48 h prior exposure. The delay in onset of action is in contrast to Dans and Isseroff (11) who found a more immediate onset of inhibition of lattice contraction by IFN-α2b and γ. This may be due to their use of neonatal fibroblasts whereas we used adult dermal and HSc fibroblasts. The delay in response of fibroblast contractility to IFN-α2b shown here resembles the effect on collagen metabolism which we have found previously (50), where at least 48 h of exposure was required and maximal effects were seen after 72 h. These findings suggest that a second messenger system may be involved and that protein synthesis may be required (36, 53, 54). Chen et al have demonstrated that the effects of gamma (type I) interferon on corneal fibroblasts are mediated by endogenous beta (type II) interferon production (42) however the signal transduction pathway of type I interferon is distinct from that of type II (21). Alpha and beta interferon share a common receptor (38) which, following ligand binding, leads to activation of a number of transcription factors which bind to regulatory sequences of interferon responsive genes (53, 54). Hence,

these IFN-inducible gene products may mediate the effects seen here following IFN-a2b treatment.

Recently, Sahara et al (44) reported that fibroblasts from keloids and HSc were more contractile than normal dermal fibroblasts obtained from different patients. However, by using paired fibroblast strains to control intersubject variability we found that hypertrophic scar and normal dermal fibroblasts from the same patient exhibit comparable abilities to induce progressive contraction. The differences may be due to the selection of the control fibroblasts chosen, emphasizing the importance of using normal dermal fibroblasts from the same patient as a control. Alternatively, differences between scar and normal fibroblasts may be lost with serial passage (50). However, we chose fibroblasts of low passage number (3-7) similar to Sahara et al (44). The number of fibroblasts and collagen concentration per lattice has also been shown to affect the rate and extent of contraction (33). The conditions used in this experiment may mask differences between the hypertrophic and normal cell strains; this aspect requires further examination.

The mechanism by which fibroblasts produce lattice contraction is not fully understood, hence the mode of action of IFN-a2b cannot be determined exactly. Of the mRNAs for the cytoskeletal proteins examined using northern blot analysis, the ß and γ isoforms of actin showed a statistically significant reduction following treatment. Smooth muscle cells treated with IFN-γ produce decreased quantities of actin mRNA, particularly the α-actin (26). This may represent a potential mechanism for the interferon-

induced reduction in contraction, as actin microfilaments are necessary for fibroblast elongation and contraction (26, 43, 48, 49). However, further investigation of actin-associated proteins is necessary, for although the level of α-actinin mRNA was not affected, other actin-associated proteins such as monomer binding, crosslinking or capping proteins also contribute to the orientation and stabilization of F-actin (31). These proteins may be affected by IFN-α2b thus accounting for the alterations in stress fibre presentation between the gels of the treated and untreated cells.

Phallotoxin has been shown to specifically bind to and stabilize actin filaments (53) which can then be visualized by fluorescent labeling (35). Although TRITC-phalloidin staining is not quantitative, it can be clearly seen in the confocal scanning laser microscope that the arrangement of actin filaments within IFN-treated and untreated fibroblasts in the collagen gels was comparable in the initial attachment stage but differed significantly in the period of active contraction. The modifications of the microfilament arrangement noted in the control fibroblasts following 24 h of contraction resemble the findings of Ehrlich et al (52), where fibroblasts with bipolar, elongated morphology and condensed actin filaments were associated with active contraction. Interestingly, the changes in stress fibre presentation noted in the fibroblasts treated for 144 h with IFN-α2b, where the lattices were contracted approximately 22% and 14% less than control HSc and normal fibroblasts respectively, are characteristic features attributed to the myofibroblast (19). Several other researchers have also detected myofibroblast-like cells in non-contracted tissues.

Doillon et al examined open wounds and burn wounds in rats where they found actin-rich fibroblasts in areas of tissue necrosis or injury, independent of the degree of contraction (49). Similarly, Squier (46) observed myofibroblasts after stretching mouse skin in vivo in the absence of wounding. Pfeffer et al (40, 41) treated fibroblasts with IFN-ß which resulted in reduced rates of DNA, RNA, and protein synthesis yet increased cell size with enlarged parallel microfilaments. Recently, Nishiyama et al (54) described morphologic alterations correlated with lattice contraction which closely resembled our own findings described here. The effect of the actin microfilament inhibitor substance cytochalasin D, led them to suggest that the morphologic changes in foreskin dermal fibroblasts (bipolarity and elongation) are closely associated with active lattice contraction, but complete disruption of actin microfilaments with low dose cytochalasin D did not prevent the acquisition and maintenance of the elongated shape of the fibroblasts in the collagen gel. These findings suggest that other cytoskeletal proteins independent of actin microfilament organization may be actively involved in the development of the morphologic features in fibroblasts which precede active contraction. However, our own data suggest that in IFN-α2b-induced inhibition of lattice contraction, distinct changes occur in actin filaments and that these changes are associated with alterations in the pattern of mRNA expression of the cytoskeletal proteins ß and γ actin, and possibly α-tubulin, but probably not vimentin or α-actinin.

In conclusion, our findings suggest that HSc fibroblasts do not appear to possess inherently superior contractile abilities relative

to their counterparts from normal dermis. The ability of cells to contract collagen gel matrices is inhibited by IFN-α2b and stimulated by increasing concentrations of serum, which may be the result of serum growth factors. The influence of IFN-α2b on the contractile rate of fibroblasts is associated with reductions in levels of mRNA for β and γ-actin. In addition, the presence of IFN-α2b affects the organization of actin filaments or stress fibres, resulting in a lack of bipolar morphology, which is characteristic of a contractile cell within the collagen gel.

3.6 Figures

Table 1   Patient demographic information. Patient 1-4's fibroblasts were used for the fibroblast-populated collagen lattice assay at 10% FBS concentration, patient 1 for the fibroblast-populated collagen lattice assay at 5% FBS concentration, patient 1 for confocal laser microscopy and patients 1-3 and 5 for Northern blot analysis. TBSA, total burn surface area; H, hypertrophic; N, normal.

Patient	Tissue	Biopsy Site	Age (yr)	Sex	TBSA	Months after burn
1	H	Neck	13	M	35	7
	N	Back				
2	H	Chest	32	M	60	4
	N	Chest				
3	H	Arm	27	F	14	14
	N	Arm				
4	H	Hand	3	M	28	6
	N	Thigh				
5	H	Hand	4	M	25	6
	N	Thigh				
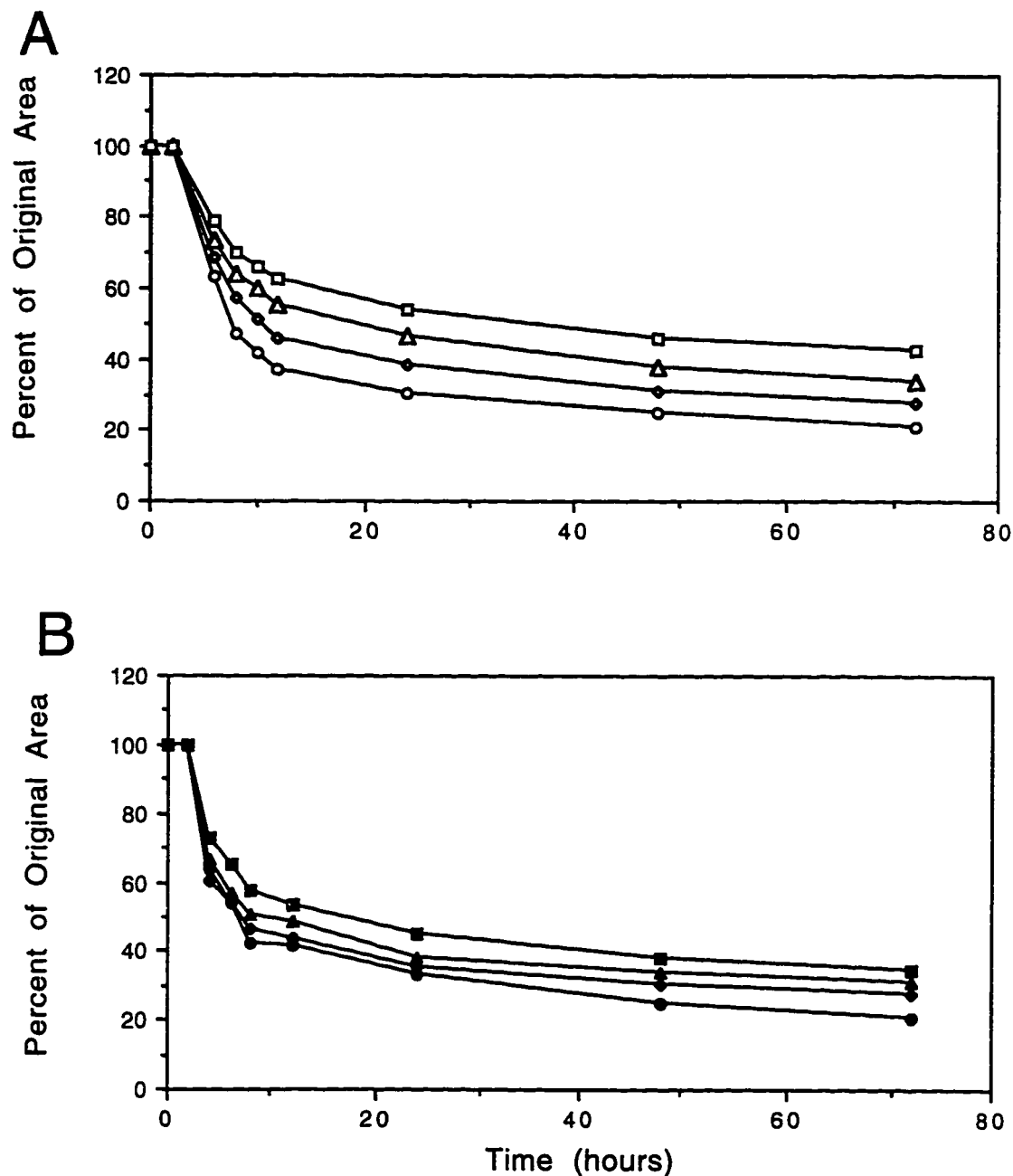
Figure 1   Contraction of collagen lattice by hypertrophic scar and normal dermal fibroblasts treated with 2000 U/ml IFN-α2b at 10% FBS concentration. A) Hypertrophic scar fibroblasts: control (o); 48 h treatment (◊); 96 h treatment (Δ); 144 h treatment (/).   B) Normal dermal fibroblasts: control (•) 48 h treatment (♦); 96 h treatment (▲); 144 h treatment (■) (p<0.05 relative to control).   Each time point represents the mean of four cell strains with three gels per cell strain (n=12).
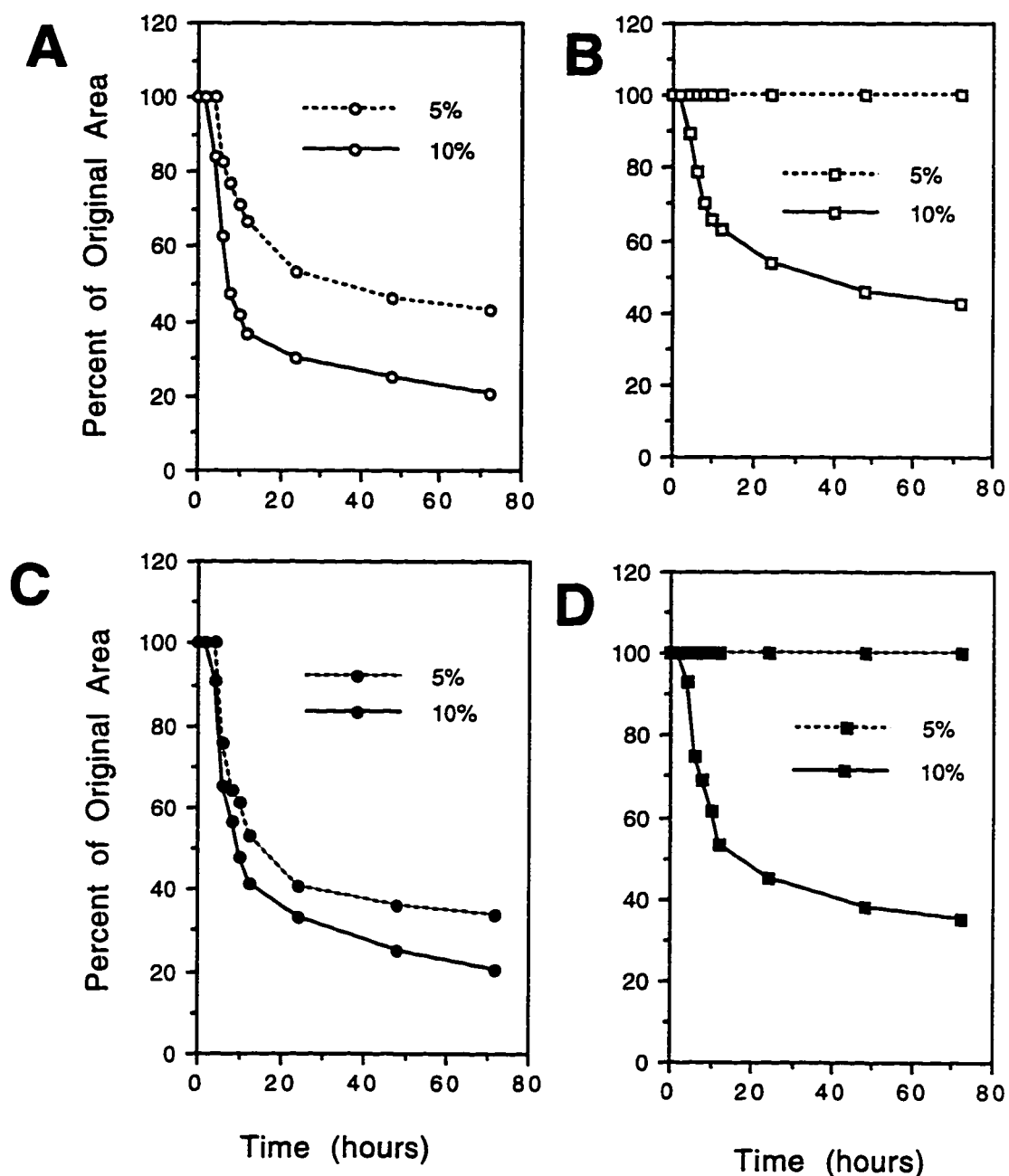
Figure 2 Fibroblast contraction at 5% and 10% FBS concentration. Fibroblasts seeded in collagen lattices with 10% FBS (*solid line*) were more contractile than those seeded at 5% FBS (*dotted line*) in all treatment groups (p<0.01). Each time point represents an average of three gels. A) Untreated hypertrophic scar fibroblasts. B) Hypertrophic scar fibroblasts treated with IFN-α2b for 144 h. C) Untreated normal fibroblasts. D) Normal fibroblasts treated with IFN-α2b for 144 h.

Figure 3   Northern analysis of γ-actin expression.   Panel A shows a typical autoradiogram hybridized with the γ-actin cDNA revealing the 2.1 kb transcript for one pair of HSc and normal fibroblast strains.   Lane H1 shows the HSc control group, lane H2 the HSc after treatment with IFN-α2b for 144 h, lane N1 the normal control group and lane N2 the normal IFN-α2b 144 h treatment group.   Panel B shows the α-actinin 3.6 kb transcript which was used as an internal control to correct for loading.   Panel C is the corresponding ethidium bromide-stained gel showing 28S and 18S ribosomal RNAs.   Panel D is a histogram of the quantitative densitometry results (corrected for α-actinin) for γ-actin mRNA in the untreated cells (solid bar) and 144 h treated cells (open bar) in four paired cell strains ±SEM (*p<0.05).
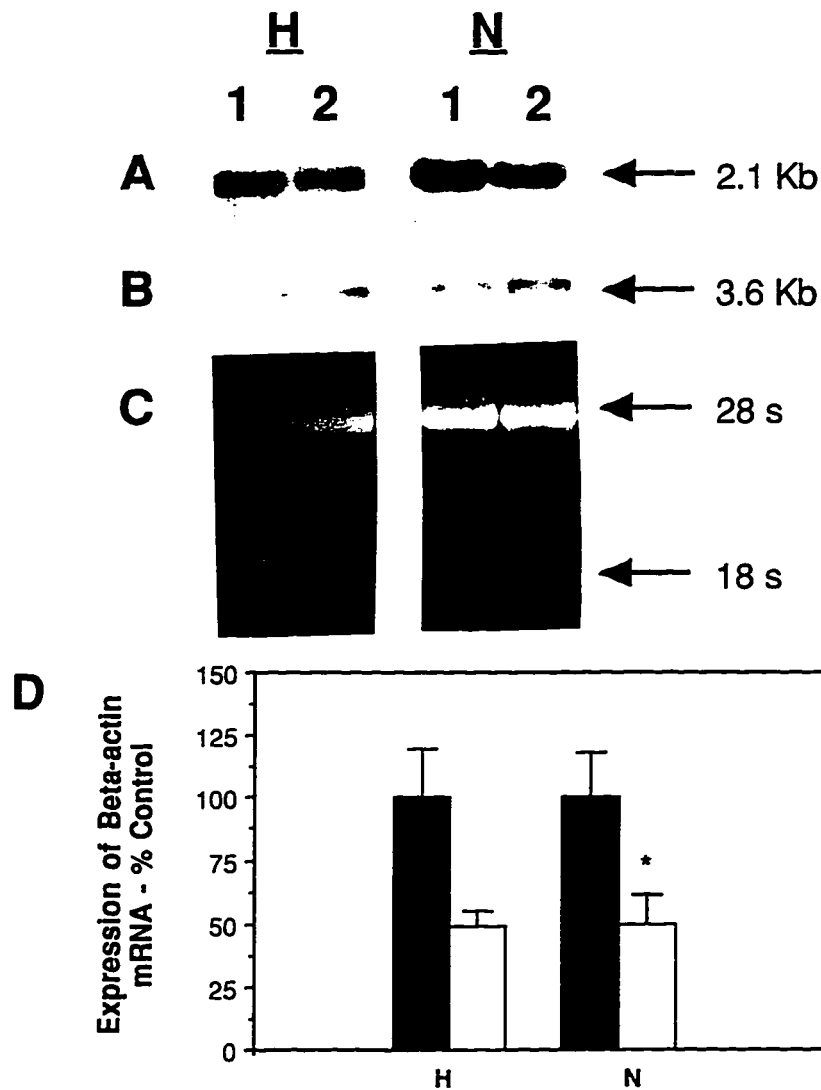
Figure 4   Northern analysis of ß-actin expression.   As indicated in Figure 3, Panel A shows a typical autoradiogram of ß-actin cDNA revealing the 2.1 kb transcript for one pair of HSc and normal fibroblast strains.   Lane H1 the HSc control group, lane H2 the HSc IFN-α2b 144 h treatment group, lane N1 the normal control group and lane N2 the normal IFN-α2b 144 h treatment group.   Panel B shows the α-actinin transcript.   Panel C is the ethidium bromide-stained gel.   Panel D is a histogram for ß-actin mRNA in the untreated cells (solid bar) and 144 h treated cells (open bar) in four paired cell strains ±SEM (*p<0.05).
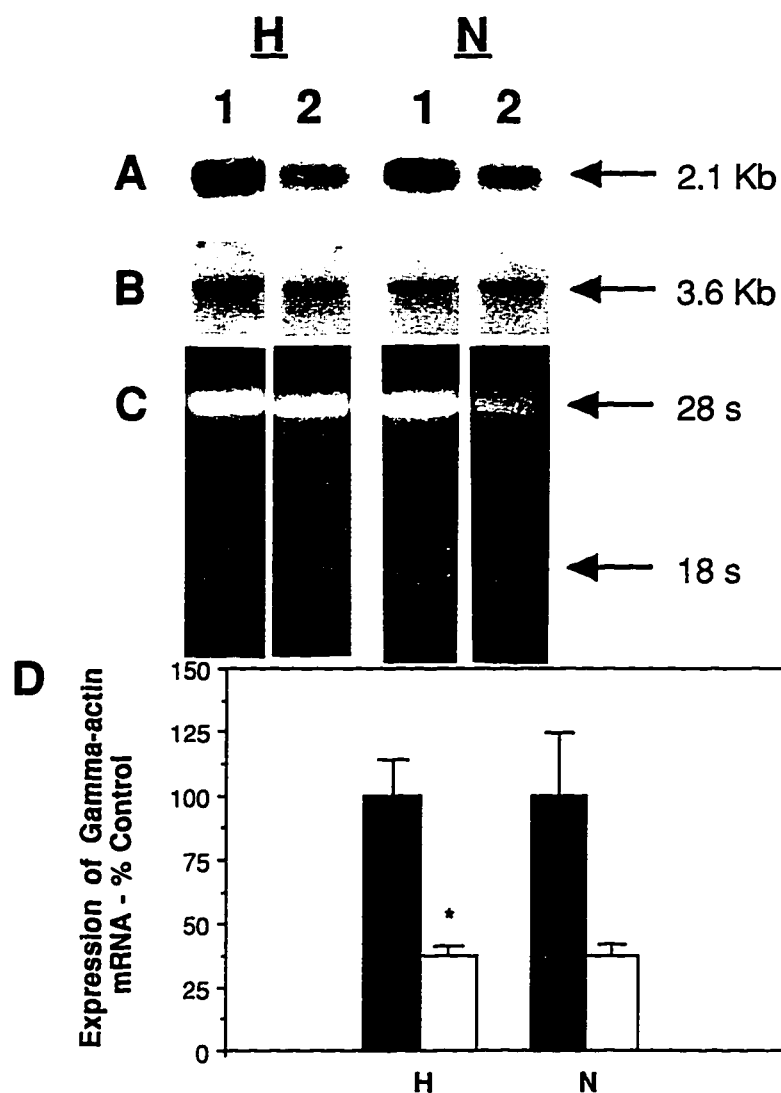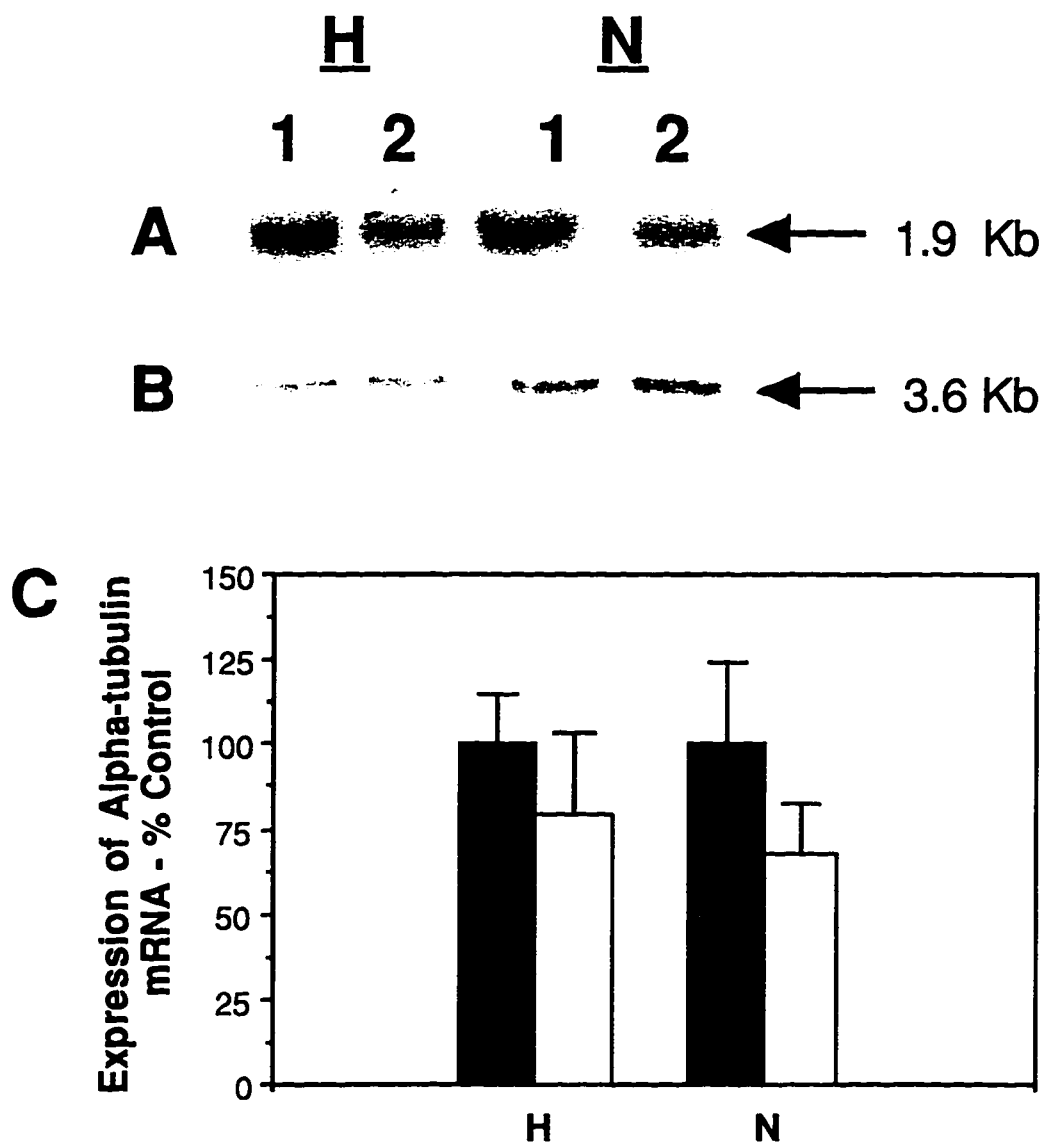
Figure 5 Northern analysis of α-tubulin expression. Panel A shows a typical autoradiogram, hybridized with the α-tubulin cDNA revealing the 1.9 kb transcript for one pair of HSc and normal fibroblast strains. Lane H1 shows the HSc control group, lane H2 the HSc IFN-α2b 144 h treatment group, lane N1 the normal control group and lane N2 the normal IFN-α2b 144 h treatment group. Panel B shows the α-actinin transcript which was used as an internal control to correct for loading. Panel C is a histogram of the quantitative densitometry for α-tubulin mRNA in the untreated cells (solid bar) and 144 h treated cells (open bar) in four paired cell strains ±SEM.
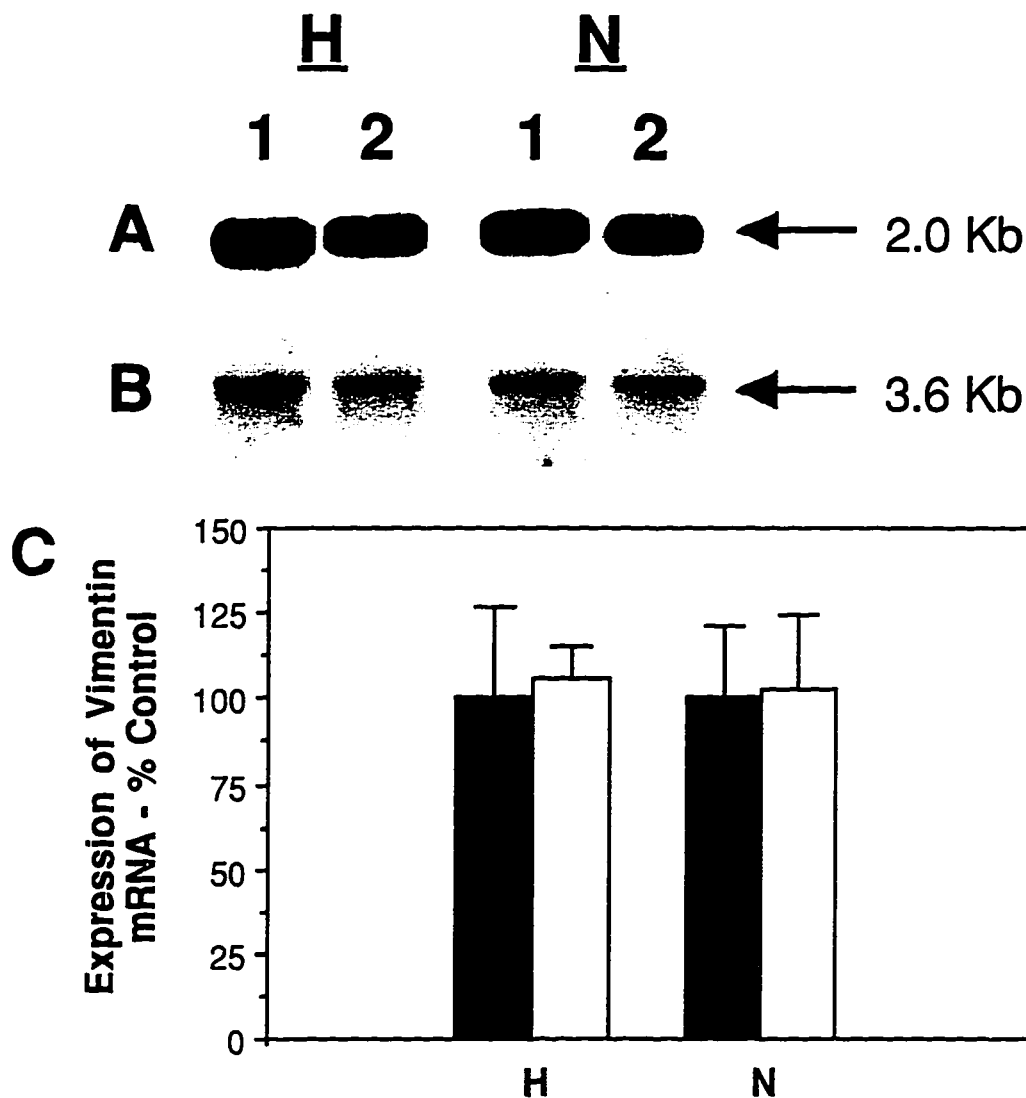
Figure 6   Northern analysis of vimentin expression.   As indicated in Figure 5, Panel A shows a typical autoradiogram hybridized with the vimentin cDNA revealing the 2.0 kb transcript for one pair of HSc and normal fibroblast strains.   Lane H1 shows the HSc control group, lane H2 the HSc IFN-α2b 144 h treatment group, lane N1 the normal control group and lane N2 the normal IFN-α2b 144 h treatment group. Panel B shows the α-actinin transcript.   Panel C is a histogram of the quantitative densitometry for vimentin mRNA in the untreated cells (solid bar) and 144 h treated cells (open bar) in four paired cell strains ±SEM.
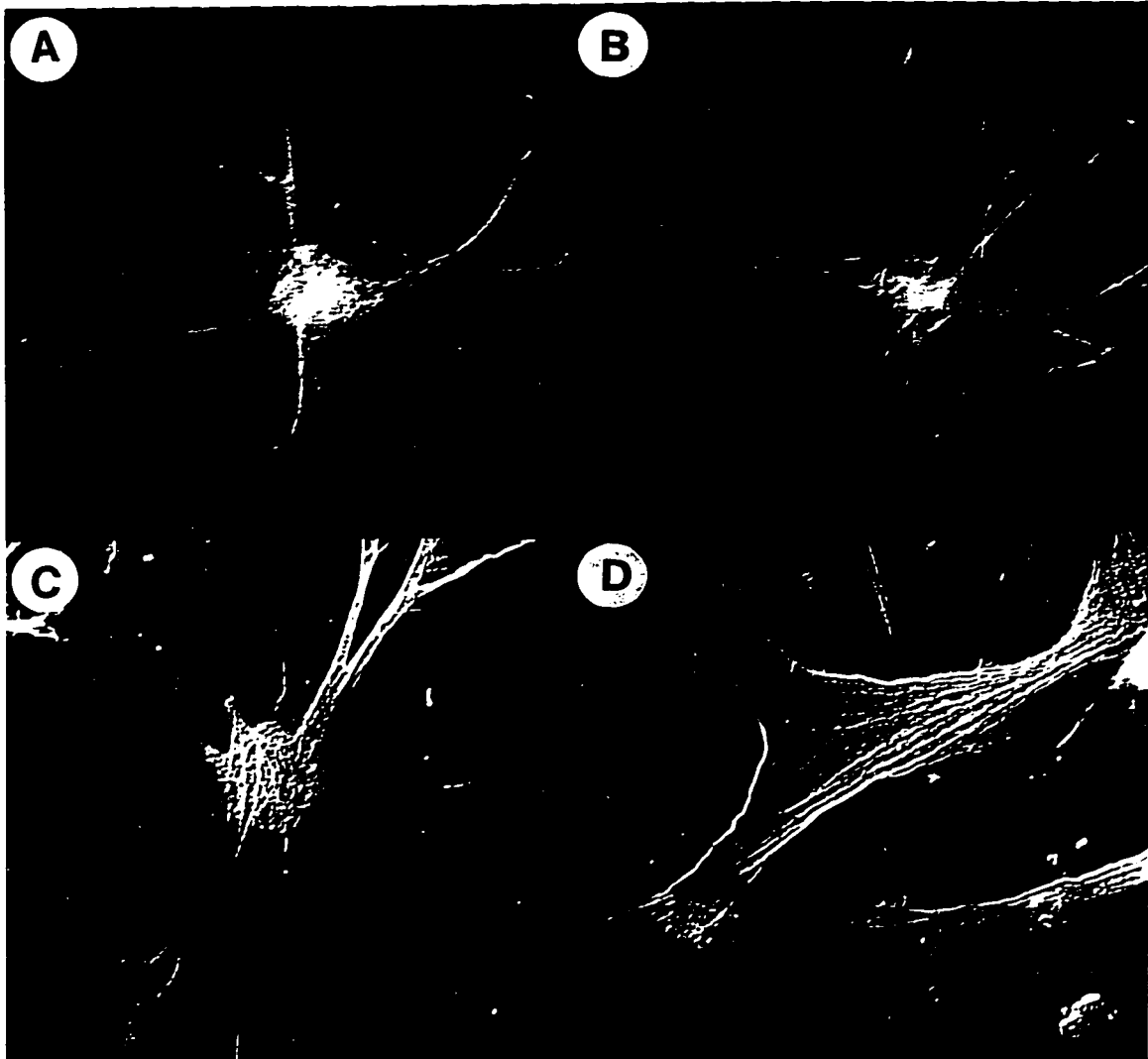
Figure 7   Confocal microscopy of normal fibroblasts in the fibroblast-populated collagen lattice.   A) Normal control fibroblasts following 2 h of contraction. B) Normal fibroblasts which had been treated with IFN-α2b for 144 h, following 2 h of contraction. C) Normal control fibroblasts following 24 h of contraction. D) Normal fibroblasts which had been treated with IFN-α2b for 144 h, following 24 h of contraction (original magnification x 400).

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CHAPTER 4:

THE EFFECT OF INTERFERON-α2b ON GUINEA PIG WOUND CLOSURE AND

THE EXPRESSION OF CYTOSKELETAL PROTEINS *IN VIVO*

4.1 ABSTRACT

Scar contraction following healing of deep partial thickness or full thickness dermal injury is a leading cause of functional and cosmetic morbidity. The therapeutic use of interferon for the treatment of fibroproliferative disorders associated with scar contraction, including hypertrophic scar, has been suggested due to its antifibrotic properties. Treatment of fibroblasts with interferon has been shown to inhibit the rate and extent of contraction using the *in vitro* fibroblast-populated collagen lattice model system. In order to establish the effect of interferon-α2b (IFN-α2b) on full thickness wound contraction *in vivo*, osmotic pumps loaded with interferon or sterile saline were implanted intraperitoneally in guinea pigs. Seven days following implantation six full thickness punch biopsy wounds were created and monitored by serial assessment of the wound area on a daily basis. Comparison between the IFN-α2b treated and control animals indicated that there was a significant reduction in the rate of wound contraction in the interferon treated animals after day three ($p < 0.01$). Western blot analysis of the tissue indicated that although the relative amount of vimentin as an indicator of mesenchymal cell number was increased after wounding ($p < 0.0001$) the myofibroblast-associated cytoskeletal proteins α-smooth muscle actin (α-SMA) and smooth muscle myosin were not. Immunohistochemistry localized the expression of α-SMA within the central region of the wound 21 days after wounding in the IFN-α2b treated animal. Staining for decorin showed a specific reduction in stain in the region that correlated

with the presence of α-SMA staining. Measurement of apoptotic cells using the TUNEL assay revealed an increase in apoptosis in the IFN-α2b treated animal at 21 days after wounding (p<0.001) which did not co-localize with α-SMA staining. Taken together these findings suggest that the inhibitory effect of IFN-α2b was not through a reduction in the myofibroblast numbers but in part through the induction of apoptosis and possibly an alteration in the relative amounts of specific extracellular matrix proteins.

4.2 INTRODUCTION

The development of scar contractures secondary to deep partial thickness or full thickness dermal wounds is a common cause of functional and cosmetic morbidity. The potential that interferon may act as an anti-fibrogenic factor has led to *in vitro* investigation of its effect in several fibroproliferative disorders. Fibroblast growth and collagen production was inhibited in normal (18) and scleroderma fibroblasts exposed to interferons (9, 19). Hypertrophic scar and normal paired fibroblasts treated with IFN-α2b demonstrated a reduction in the rate of fibroblast proliferation and fibronectin, type I and type III collagen mRNA (14, 42). Additionally, IFN-α2b enhances the production of collagenase both at the level of mRNA and protein (13) which may be a significant contributing factor in the remodeling of wound tissue. Subsequent studies using the fibroblast-populated collagen lattice model system which simulates wound contraction (1) determined that interferon-α, -ß, and -γ (3, 26, 36) reduced the rate and extent of

contraction. However, the *in vivo* environment is far more complex with many factors that may alter the effect of interferon on fibroblasts ability to attach to and rearrange the extracellular matrix.

The specific mechanism by which wound contraction occurs is still not well understood. It has been proposed that a cell which has features of both a fibroblast and a smooth muscle cell is responsible for wound contraction (22). The term myofibroblast was coined when it was shown that one of the unique features of this cell was an increase in microfilament bundles or stress fibers similiar to those in smooth muscle cells (11). The designation of a myofibroblast has evolved into a fibroblast that expresses α-smooth muscle actin (α-SMA), although variations in the expression of other cytoskeletal proteins, including intermediate filament proteins and myosins, have also been described (6). Although the effect of IFN-α2b on α-SMA expression has not been examined, it has been demonstrated that IFN-γ reduces its expression in smooth muscle cells and fibroblasts *in vitro* (8, 16). Intralesional injection of IFN-γ for the treatment of hypertrophic scars and Dupuytren's disease demonstrated a local reduction in the myofibroblast numbers as assessed by immunohistochemistry (29).

In the present study we have examined the effect of IFN-α2b on full thickness wound contraction *in vivo*. Analysis of the wound tissue using Western blot analysis, immunohistochemistry and *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed to evaluate the expression of various cytoskeletal proteins during the course of wound healing

as well as the effects of IFN-α2b. Our results indicate that IFN-α2b inhibits wound contraction. Evaluation of myofibroblast cytoskeletal proteins suggested that the inhibitory effect of IFN-α2b was not through a reduction in the myofibroblast population but may be through the induction of apoptosis and/or an alteration in the composition of the extracellular matrix.

4.3 MATERIALS AND METHOD

In Vivo Experiments

Hartley guinea pigs weighing approximately 350 grams were used for all experiments following a two week conditioning period. Seven days prior to wounding the guinea pigs were randomly assigned to control and treatment groups. They were premedicated with 0.05 mg/kg of buprenorphine then anesthetized by nose cone using methoxyflurane. The abdomen was clipped, washed with a mild soap and disinfected. Osmotic pumps (Alzet model 2ML4, Palo Alto, CA) loaded with sterile saline or IFN-α2b were then implanted intraperitoneally in the control or treatment animals respectively. The weight of the guinea pig and the manufactures specification that the pump delivers 2.5 μl/hour were used to calculate the concentration of IFN-α2b required to deliver 3×10^6 U/m²/day to the individual guinea pigs. On day 0 the guinea pigs were again premedicated and anesthetized, their backs clipped, depilated, washed and disinfected. Using 8 mm disposable punch biopsies (Dormer Laboratories Inc Toronto, Canada), six full thickness wounds

were created, three on each side with at least 2 cm separating the wounds. Any bleeding was controlled with pressure and a polyurethane dressing was applied (Opsite™).

Wound contraction was measured daily using a direct transparency overlay to trace the wound circumference. The tracings were later scanned and quantitated using the NIH Image processing and analysis program for Macintosh.

Pairs of animals were sacrificed on days 7, 14 and 21 at which time tissue samples were collected from the wound sites using 8 mm punch biopsies and stored at -80° C for Western Blot analysis or in 4% paraformaldehyde for immunohistochemistry. The osmotic pumps were also retrieved at the time to ensure that the expected amount of IFN-α2b had been delivered. Throughout the experiments all animals were cared for in accordance with the guidelines of the Health Sciences Animal Welfare Committee, and the guidelines of the Canadian Council on Animal Care.

Materials

Biotin-16-2'-deoxyuridine-5'-triphosphate, 4-nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate and terminal deoxynucleotidyl transferase (TdT) were obtained from Boehringer Mannheim (Indianapolis, IN). RNase-free DNase was obtained from Promega (Madison, MI). Bicinchoninic acid (BCA) protein assay reagent and Supersignal™ CL-HRP Substrate System were obtained from Pierce (Rockford, IL). Casein blocking buffer was obtained from Cambridge Research Biochemicals (Northwich,

Cheshire, UK). Anti-alpha smooth muscle actin (clone 1A4), anti-actin (clone AC-40), anti-desmin (clone DE-U-10), anti-smooth muscle myosin (clone hSM-V), anti-vimentin (clone V9) monoclonal antibodies, ExtrAvidin®-Alkaline Phosphatase, normal goat serum, phenylmethylsulfonyl fluoride (PMSF), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), ethyleneglycol-bis-(ß-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), leupeptin, pepstatin and aprotinin were obtained from Sigma Chemical Company (St Louis, MO). Skeletal myosin heavy chain was detected using the IgG1 monoclonal antibody, clone MY-32 obtained from Zymed Laboratories Inc (San Franscisco, CA). Non-muscle myosin antibody developed in rabbit was obtained from Biomedical Technologies Inc (Stoughton, MA). Decorin was detected using the IgG monoclonal antibody 6D6 (30).

Western Blot Analysis

Tissue samples from normal skin tissue and the wounds after 7, 14 and 21 days of healing were extracted using a two step procedure (41). Frozen tissue samples were minced into small pieces while on ice. Tissue was then homogenized using a Polytron™ (Brinkmann Instruments, Ont, Canada) in 2 ml of solution A (10 mM Tris-acetate, 6 mM 2-mercaptoethanol, 1 mM EGTA, 4 mM MgCl₂, 0.5% Triton-X 100, 1 mM PMSF, 1 mM TPCK, leupeptin, pepstatin, aprotinin and sodium azide) for 2 min while on ice. Samples were centrifuged for 5 min at 4° C at 1000 x g. The supernatant was discarded and the precipitate was rehomogenized 4 times as

indicated above. The precipitate was then homogenized in 2 ml of solution B (3 mM EDTA, 6 M urea, 10 mM Tris-acetate, 6 mM 2-mercaptoethanol, TPCK, PMSF, leupeptin, pepstatin, aprotinin and sodium azide) for 2 min, Mg^{2+} adjusted to 5 mM, centrifuged at 4° C for 5 min at 37,000 x g, the supernatant collected and EDTA concentration adjusted to 6 mM. The supernatants were then exhaustively dialyzed against ddH_2O, frozen and lyophilized.

Samples were resuspended in running buffer and the protein content was measured using the BCA protein assay system. Samples were run on an 8% SDS polyacrylamide gel as described (24) and transferred to nitrocellulose. The membranes were blocked with 2% non-fat milk at 37° C for 30 min, then probed with the primary antibody of interest at a dilution of 1:500 for 2 hours at room temperature. The antibodies used were for vimentin, total actin, alpha smooth muscle actin, desmin, smooth muscle myosin and non-muscle myosin. The membranes were washed four times with PBS/Tween then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase at a dilution of 1:1000 for 1.5 hours at room temperature. The membranes were developed using a chemiluminescence system and exposed to X-ray film. When comparisons were made between different proteins the membranes were stripped by incubating with stripping buffer (2% w/v SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM ß mercaptoethanol) for 30 minutes at 37° C. The membranes were then washed with PBS and exposed to film to ensure that the blot had been completely stripped of the previous signal. The membrane was then re-probed for the second

protein of interest as described above. This procedure was repeated for up to three proteins per membrane.

The autoradiograms were then scanned and quantitated using the NIH Image v1.59 processing and analysis program for Macintosh.

Immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4-5 μm sections which were placed on 3-aminopropyl-triethoxy-silane coated slides. The sections were then stained for the proteins of interest using the horseradish peroxidase/diaminobenzidine (DAB) development system to allow for localization of protein expression at 7, 14, and 21 days following wounding.

All sections were heated to 60° C for 10 min, dewaxed in xylene then rehydrated. Endogenous peroxides were inactivated by treating all sections with 10% H_2O_2 in methanol for 6 min. After washing with PBS, sections were incubated for 30 min at room temperature in blocking agent (10% v/v normal goat serum, 5% bovine serum albumin w/v in PBS) and then incubated overnight at 4° C with the appropriate antibody. The antibodies were diluted in PBS/Tween 20 0.05% at a 1:100 ratio for α-SM actin and 1:10 for decorin. Controls for the monoclonal antibodies used plasmacytoma cell culture supernatant. The sections were incubated with goat F(ab')2 fragment to mouse IgG (whole molecule; 1:50 dilution in PBS/Tween 0.05%) for 1.5 hours, washed with PBS, then incubated with monoclonal peroxidase-anti-peroxidase soluble complex at a

1:200 dilution for 1.5 hours and washed with PBS. The colorimetric reagent was hydrogen peroxide/DAB and all sections were counterstained with haematoxylin.

TUNEL Assay

The *in situ* demonstration of DNA fragmentation was performed using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) as previously described (12). All sections were treated in the same manner as described for immunohistochemistry up to the point of rehydration. After washing with ddH2O, the sections were incubated for 20 min at 37° C in 2 x sodium chloride and sodium citrate (SSC) (pH 7.0) then washed several times in ddH2O. All sections were immersed for 5 min at room temperature (RT) in 10 mM Tris-HCl (pH 8.0) then digested for 15 min at 37° C with proteinase K (20 μg/ml in 10 mM Tris-HCl). After washing several times in ddH2O the positive control was incubated in DNase buffer for 5 min at RT then digested with DNase (1 μg/ml) at 37° C for 30 min. The positive control was washed with ddH2O then all sections were incubated for 5 min at RT in TdT buffer (pH 7.2). All sections were incubated in reaction buffer (94.5 μl ddH2O, 7.0 μl TdT buffer, 4.4 μl Bio-16-dUTP, 4.4 μl dUTP and 1.5 μl TdT) for 1 hour at 37° C in a humidified chamber, except the negative control where the TdT was omitted. To terminate the reaction the sections were transferred to 2 x SSC for 15 min at RT. All sections were washed with PBS then incubated for 15 min at RT in blocking agent (1:5 casein blocking buffer, 10% v/v normal goat

serum, and 5% bovine serum albumin w/v in PBS). The sections were then incubated at RT for 30 min in Extra-Avidin phosphatase (1:250) in PBS/Tween 0.5%. The sections were washed with PBS then incubated with colorimetric buffer (0.1 M Tris, 0.1 M NaCl, 0.25 M MgCl$_2$; pH 9.5) for 5 min at RT. The colorimetric reagent was 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

The percentage of positive cells was calculated by counting the number of positive cells in 10 randomly selected fields within the wound tissue and comparing it to the total number of cells within the correlating serial section which was stained with haematoxylin.

Statistics

Student's two-tailed paired t test was used for all comparisons. Results are reported using p value, mean and standard error of the mean.

4.4 RESULTS

Effect of IFN-α2b on Wound Contraction *in vivo*

Wound contraction was monitored on a daily basis and measurements converted to the percent of the original surface area on day one following the creation of full thickness wounds. When the osmotic pumps were implanted one week prior to wounding,

there was a significant reduction in the rate and extent of contraction following day three in those guinea pigs treated with IFN-α2b (Figure 1). Wound closure occurred on approximately day nine for all animals. A visible difference in the size of the scar remained at a macroscopic level and microscopically when the tissues were sectioned but since it is technically difficult to ensure that the sections were taken at the same point in the wounds, these observations were not quantitated.

A pilot study was conducted where the osmotic pumps were implanted on the same day that the wounds were created but no significant difference was seen in the contraction rate (data not shown). This suggested that prior exposure to interferon was required to inhibit contraction. The guinea pigs were weighed throughout the experiment and showed a gradual increase during the preconditioning period and the experimental period with slight losses following procedures such as implantation of osmotic pumps and wounding. There was no significant difference in the weights of the IFN-α2b treated animal relative to the controls (Figure 2).

Total Expression of Cytoskeletal Proteins

In an attempt to establish how interferon inhibits wound contraction, animals were euthanized at 7, 14 or 21 days after wounding and samples of the wound were obtained using an 8 mm punch biopsy. Cytoskeletal proteins from two different wound sites per animal were extracted and Western blot analysis performed. Vimentin showed a significant increase following wounding in all

groups suggesting that there was an increase in the number of mesenchymal cells, which predominantly consisted of fibroblasts (p<0.0001) (Figure 3).

Since it has been suggested that fibroblasts evolve into myofibroblasts which can express altered levels of cytoskeletal proteins including actins, myosin and intermediate filament proteins (6), several cytoskeletal proteins were compared to vimentin as a index of fibroblast numbers. Total actin, α-SMA and smooth muscle myosin were all significantly reduced relative to vimentin in the tissue following wounding compared to normal tissue from the same animal (Figure 4A). When α-SMA was compared to desmin as an index of smooth muscle cell content there was a 40% reduction in α-SMA (Figure 4B). When comparisons were made to vimentin as a function of time by dividing the data into the three groups being 7, 14, and 21 days there was a significant increase in α-SMA and smooth muscle myosin at 14 days relative to 7 days (Figure 5). The increase in α-SMA and smooth muscle myosin indicated that there may be an increase in myofibroblasts between 7 and 14 days after wounding with a reduction at 21 days. To localize putative myofibroblasts within the tissue staining for α-SMA was performed (see below).

To evaluate the effect that interferon treatment had on the relative quantity of cytoskeletal proteins, comparisons were made between the tissue from the animals which were treated with IFN-α2b relative to the control group. For all cytoskeletal proteins examined (Figure 6).

Localized Expression of α-Smooth Muscle Actin and Decorin

Immunohistochemical staining for α-SMA showed positive staining at the base of the hair follicles as previously reported (32) and of the smooth muscle cells of the vessels in all of the sections. The wounds at 7 days had very few fibroblasts that stained positive for α-SMA (data not shown). The wounds at 14 days showed a small number of faintly staining fibroblasts in the central region of the wound but no difference could be seen between the saline control animals and those treated with IFN-α2b (data not shown). The wounds at 21 days however showed intense staining of fibroblasts in the center of the wounds from the IFN-α2b treated animals but little or no staining in the control animals (Figure 7).

The wounds were also probed for the presence of decorin. Interestingly there was weak staining for decorin using 6D6 antibody in the region where α-SMA stained strongly in the IFN-α2b treated animal 21 days following wounding yet intense staining existed throughout the wound of the control animal (Figure 8).

Localization of Apoptotic Cells

To determine whether one of the mechanisms by which IFN-α2b inhibits contraction is through induction of apoptosis, in situ TUNEL was performed. In addition, since the expression of α-SMA has been correlated with apoptosis (7) the presence of positive staining for α-SMA staining may indicate the induction of apoptosis. Positive staining was only seen in a small percentage of cells 7 days after

wounding for either the control or the IFN-α2b treated animals (4.05%±0.97 and 2.28%±0.63). There was an increased number of positive cells in the 14 day wounds which were comparable between the control and IFN-α2b treated animals (10.6%±1.83 and 12.02%±1.91). At 21 days after wounding there were substantial increases in positive cells within the IFN-α2b treated animals relative to the control animals (43.1%±19.75 vs 9.47%±6.11; n=10) (p<0.001) (Figure 9A, 9B and 9E). The location of the positive cells however was predominantly at the base of the wound and through the lower central region as opposed to the upper wound region where the α-SMA staining was located. There were no positively stained cells in the control sections where TdT was eliminated from the reaction buffer (Figure 9C) and the positive control, which was DNase digested, showed positive cells throughout the wound (Figure 9D).

4.5 DISCUSSION

It is well established that the predominant action by which most animals heal full thickness wounds is through contraction (28). Although human wound healing is achieved to a lesser extent through contraction (2, 31) the pathologic development of scar contractures is believed to be a fibroblast based process with similar features (34). Our results suggest that full thickness wound contraction is significantly inhibited in a guinea pig model following systemic administration of IFN-α2b. Although the results are not as dramatic as those seen *in vitro* (3, 15, 26, 36), the effect of additional variables such as serum binding proteins, endogenously produced

cytokines and extracellular matrix components produced by other local cells and the fibroblasts themselves will temper the biologic effect which IFN-α2b has on fibroblasts. The requirement for exposure to IFN-α2b prior to inhibition of contraction being demonstrated, suggests that new protein formation or preconditioning of cells is required. Similiar delays in effect were seen when treating fibroblasts *in vitro* with IFN-α2b (42) as compared to IFN-γ (17).

In an attempt to establish the mechanism by which IFN-α2b may have inhibited wound contraction the expression of α-SMA was investigated. Previous reports that IFN-γ reduced the expression of α-SMA *in vitro* in both arterial smooth muscle cells and fibroblasts (8, 16) suggested that IFN-α2b may have inhibited wound contraction by down-regulating the expression of α-SMA. The increased proportion of vimentin in the tissue after wounding supports the well established paradigm that fibroblast proliferation occurs during the granulation tissue formation phase of wound healing (25). The dramatic decrease in α-SMA and smooth muscle myosin relative to vimentin does not support the supposition that for contraction to occur fibroblasts require an increased expression of contractile proteins that are characteristic of smooth muscle cells (21, 23). However, examining protein quantities from tissue homogenates does not allow for precise discrimination between various cell types. The comparison of α-SMA relative to desmin as an indicator of differentiated smooth muscle cells (20, 40), establishes that the ratio remains either unchanged or lower after wounding thereby ruling out the possibility that the decrease in α-SMA following

wounding was attributable to smooth muscle cells. The peak in expression of α-SMA and smooth muscle myosin at day 14 suggests that there was an increase in the myofibroblast population relative to day 7 as has previously been shown in a rat model (5). The reduction at 21 days however did not reflect the *in situ* immunohistochemistry. In addition there was no detectably significant difference between the α-SMA content of the control animals and the treatment animals

Positive staining of fibroblasts for α-SMA at 21 days in the IFN-α2b-treated animal wound was somewhat unexpected in that the time of rapid reduction in wound area is between three and seven days and the IFN-α2b treated wounds were less contractile, both of which would make the presence of myofibroblasts less likely if it is presumed that myofibroblasts correlate with contraction. The increased proportion of apoptotic cells in these tissue samples however suggests that the expression of α-SMA may correlate with the induction of apoptosis. The induction of apoptosis by IFN-α has been demonstrated in several cell lines including activated T cells, squamous cell skin cancer cells and myeloma cells (4, 10, 33) and may partially account for the reduction in wound contraction following IFN-α2b treatment. To our knowledge evidence for the induction of apoptosis in fibroblasts by IFN-α2b has not been previously reported.

The altered production of extracellular matrix proteins following interferon treatment was the antifibrotic effect which originally led to the suggestion that interferons may be of therapeutic value in the treatment of fibroproliferative disorders.

The lack of decorin staining in the wound region that correlates with the α-SMA staining implies that the presence of myofibroblasts and the reduction in contraction may not simply be dictated by the expression of cytoskeletal proteins but also by the fibroblasts ability to reorganize the extracellular matrix proteins. It has been suggested that decorin plays a role in collagen fibril assembly into fibres (37). The lack of decorin staining is specifically located in regions of the tissue where collagen fibrils rather than fibre bundles are present. The lack of decorin within hypertrophic scar and the return to more normal levels in mature scar has previously been reported (38, 39) and suggests that its presence is required for normalization of collagen organization. The expression of α-SMA within fibroblasts may be a reflection of their inability to facilitate collagen fibre formation and hence become terminally differentiated, similiar to the α-SMA expression in smooth muscle cells that are quiescent (27). The observation that decorin staining was present at 7 and 14 days after wounding yet is reduced at 21 days suggests that during the dynamic remodeling of scar the degradation of proteoglycans in addition to collagen may significantly contribute to its reorganization. As the tissue is remodeled it may also disrupt the ability of cells to attach to the extracellular matrix which in turn may contribute to enhanced levels of apoptosis (35).

4.6 Figures
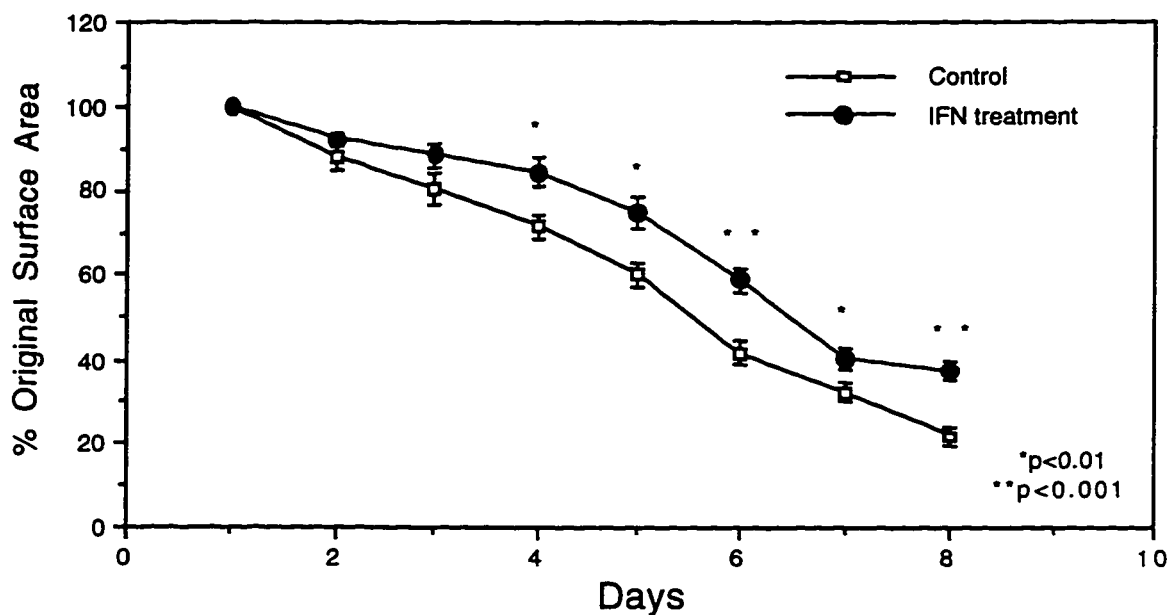
Figure 1 IFN-α2b inhibits the rate and extent of wound contraction. Full thickness wounds created on the dorsum of guinea pigs were measured on a daily basis. The guinea pigs had osmotic pumps filled with sterile saline or IFN-α2b were implanted intraperitoneally. The graph depicts the reduction in surface area of the wound relative to the original size on the first day after wounding which shows a significant reduction in wound contraction of the IFN-α2b treated animals following day three at a p value of <0.01 or <0.001 as indicated (n=18).
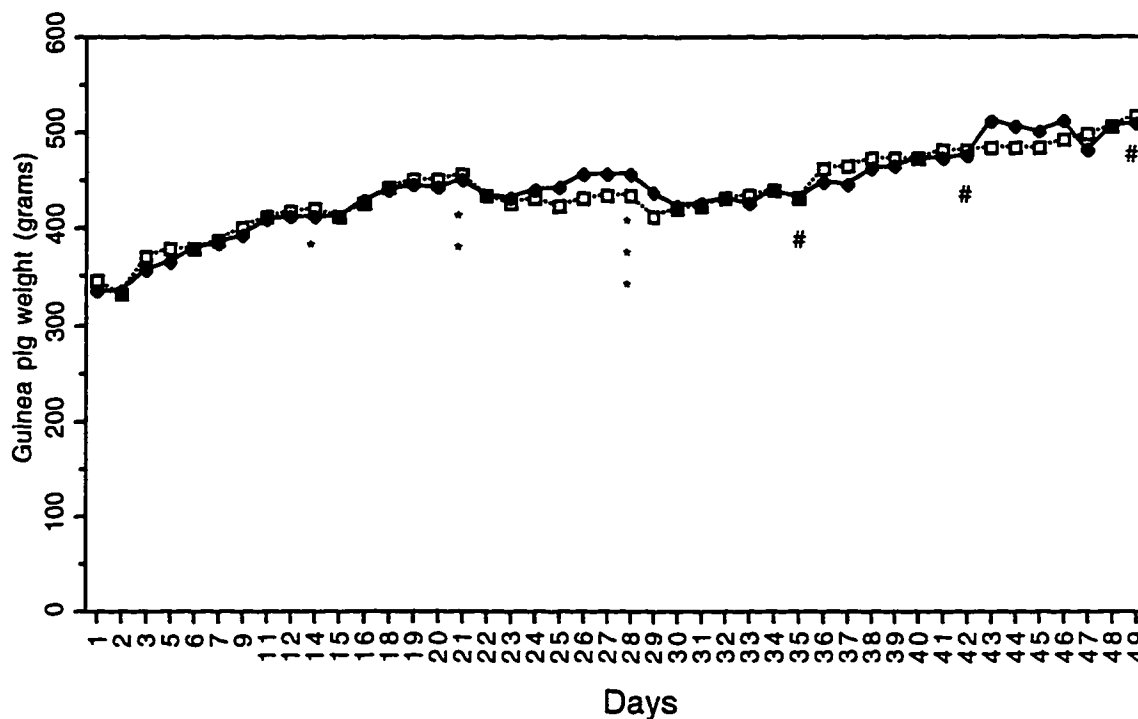
Figure 2 The weight of the guinea pigs throughout the experimental period. The weight of the guinea pigs did not differ significantly between the control animal and the interferon treated animals (dotted line and solid line respectively). The day that the pretreatment blood samples were taken is indicated by one asterisk (*), the implantation of osmotic pump by two (**), and the creation of full thickness wounds by three (***). Control and treatment animals were euthanized 7, 14, and 21 days following wounding as indicated by the number symbol (#).

A   prewounding / after wounding

I_____I I_____I I_____I
     7 d   14 d   21 d

B
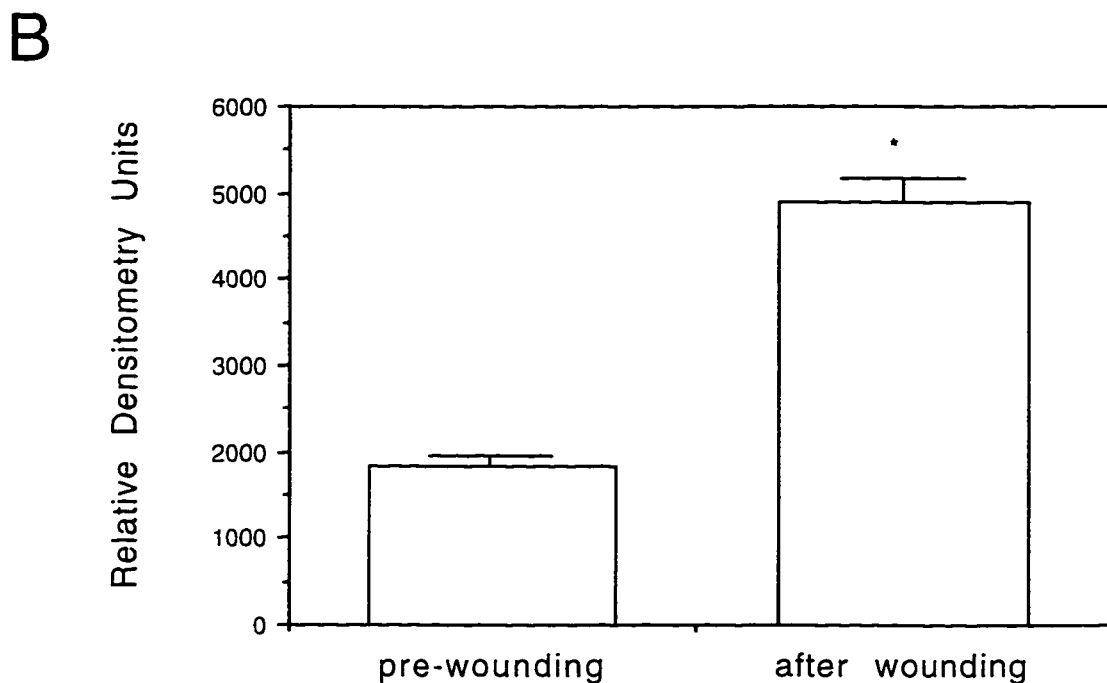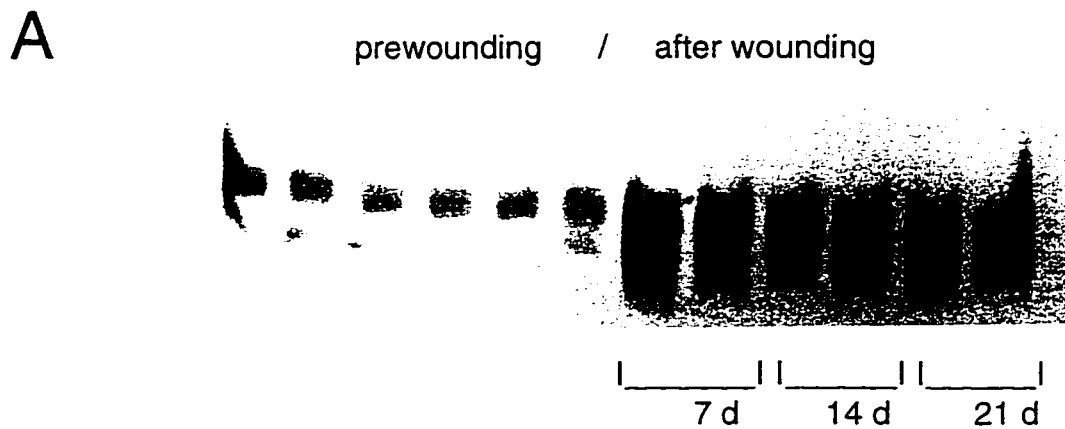
Figure 3   Vimentin was significantly increased following wounding. Panel A depicts an autoradiogram of a Western blot probed for vimentin.   The first six lanes are tissue extracted from normal tissue and the second six lanes tissue extracted following wounding as indicated.   Panel B graphically depicts the relative proportions of vimentin in the pre-wounding and after wounding tissue extraction samples (*p<0.0001) (n=24).
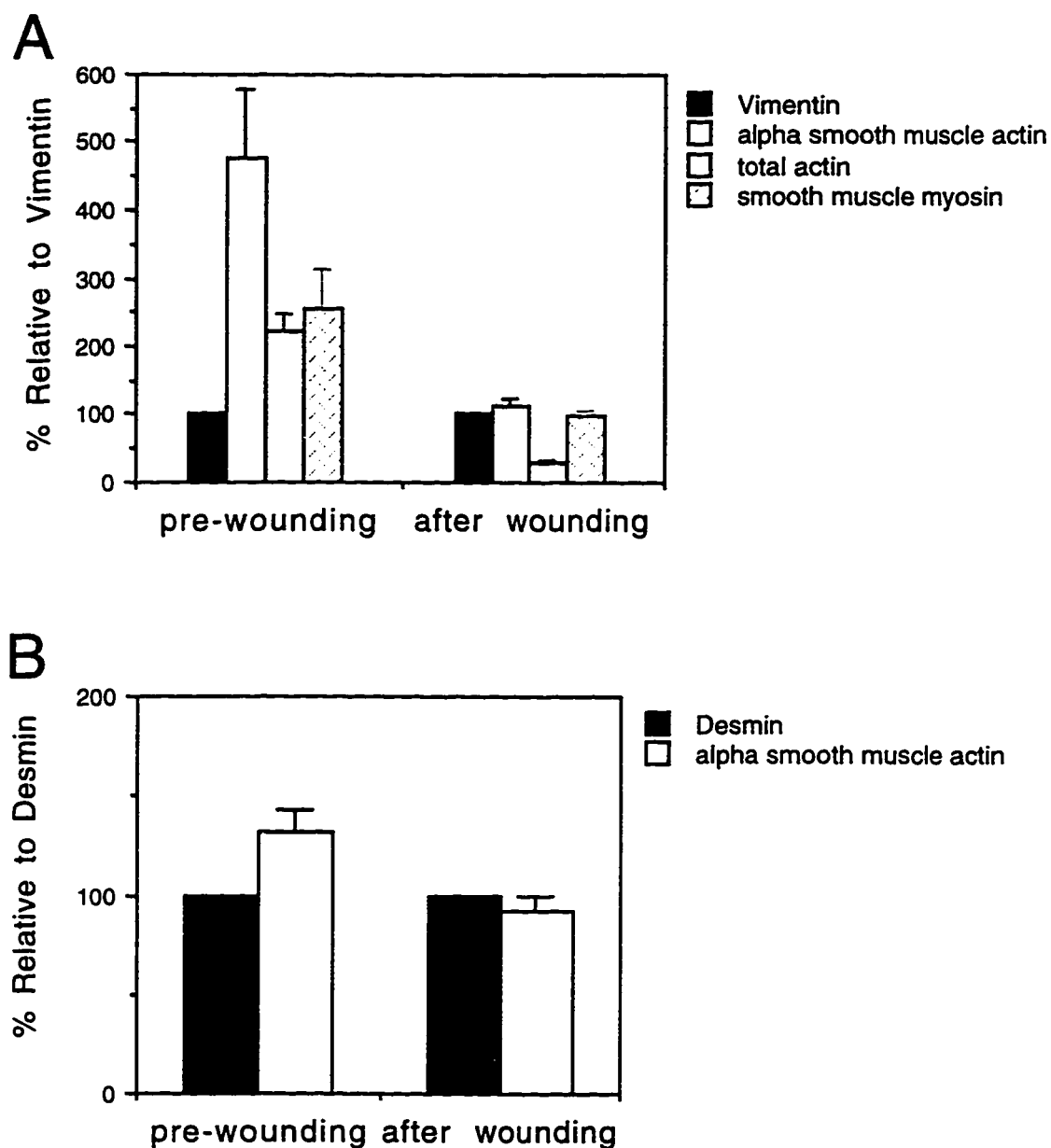
Figure 4   Cytoskeletal protein content relative to vimentin and desmin. Panel A compares the relative expression of alpha smooth muscle actin, total actin and smooth muscle myosin to vimentin which is an indicator of total fibroblasts within the tissue. All cytoskeletal proteins show a decrease relative to vimentin after wounding as compared to pre-wounding (n=12). Panel B compares the relative expression of alpha smooth muscle actin to desmin which is an indicator of total smooth muscle cells. Alpha smooth muscle actin decreases slightly relative to desmin after wounding as compared to pre-wounding.
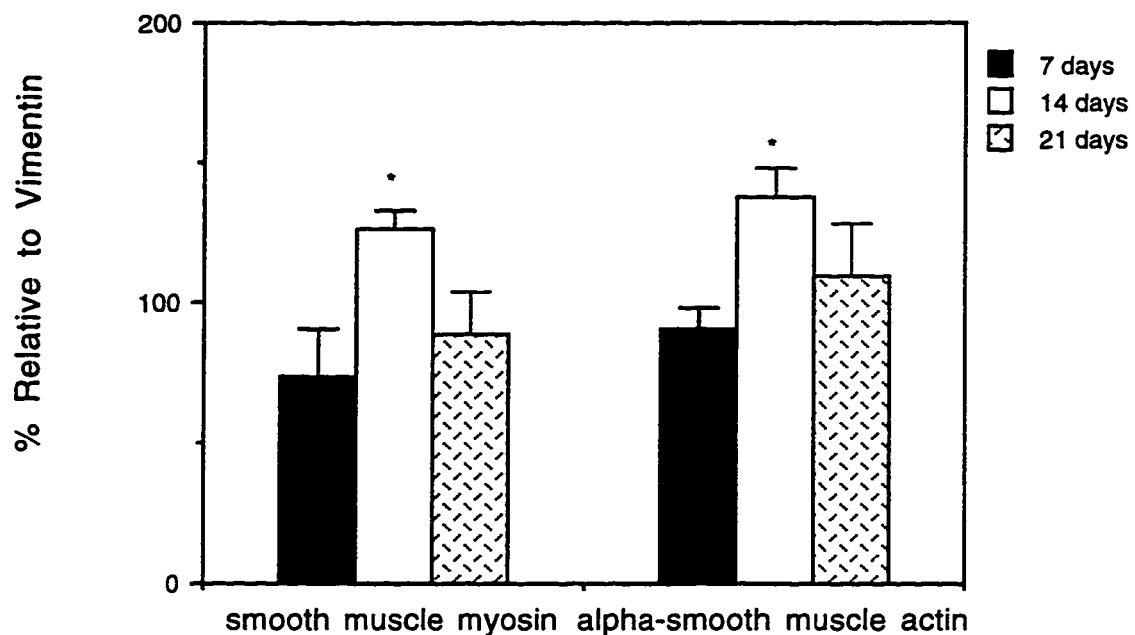
Figure 5 Comparisons of smooth muscle myosin and actin relative to vimentin at varying time points. Relative to vimentin, smooth muscle myosin and alpha smooth muscle actin significantly increase between day 7 and day 14 (p<0.001; n=4) where 1 is smooth muscle myosin and 2 is alpha smooth muscle actin.
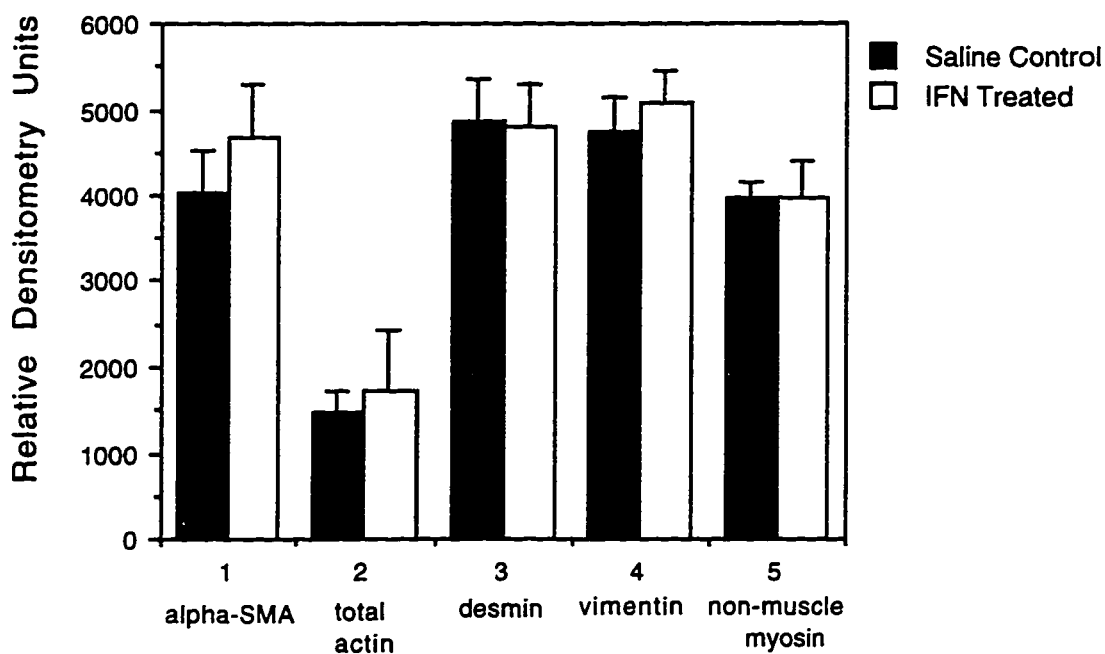
Figure 6 Comparison of cytoskeletal proteins from wound tissue of control animals and IFN-α2b treated animals. Comparisons of 1) alpha smooth muscle actin, 2) total actin, 3) desmin, 4) vimentin, and 5) non-muscle myosin showed no significant difference when tissue extracted from saline treated control animals were compared with IFN-α2b-treated animals (n=6).
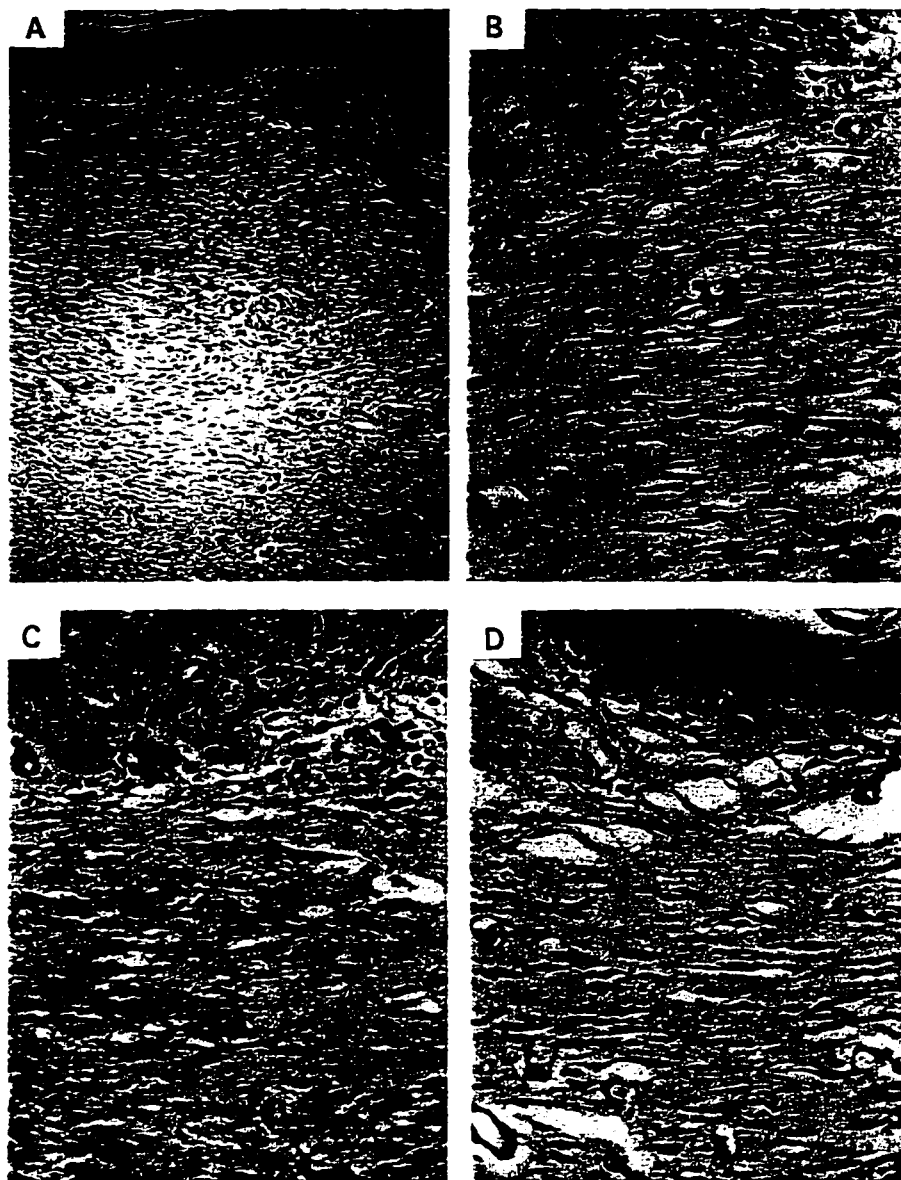
Figure 7   Alpha smooth muscle actin staining in IFN-α2b treated wounds and control wounds 21 days after wounding. Panel A shows a 50x magnification of the IFN-α2b treated wound at 21 days which was stained for alpha smooth muscle actin. Positive staining can be seen throughout the entire central upper region of the wound. Panel B shows the staining control at 100x magnification where the primary antibody was substituted with plasmacytoma cell culture supernatant. Panel C shows the IFN-α2b treated wound as shown in panel A at 100x magnification which demonstrates that it is cell specific staining. Panel D shows the control wound stained for alpha smooth muscle actin which shows positively stained smooth muscle cells but no fibroblast staining.
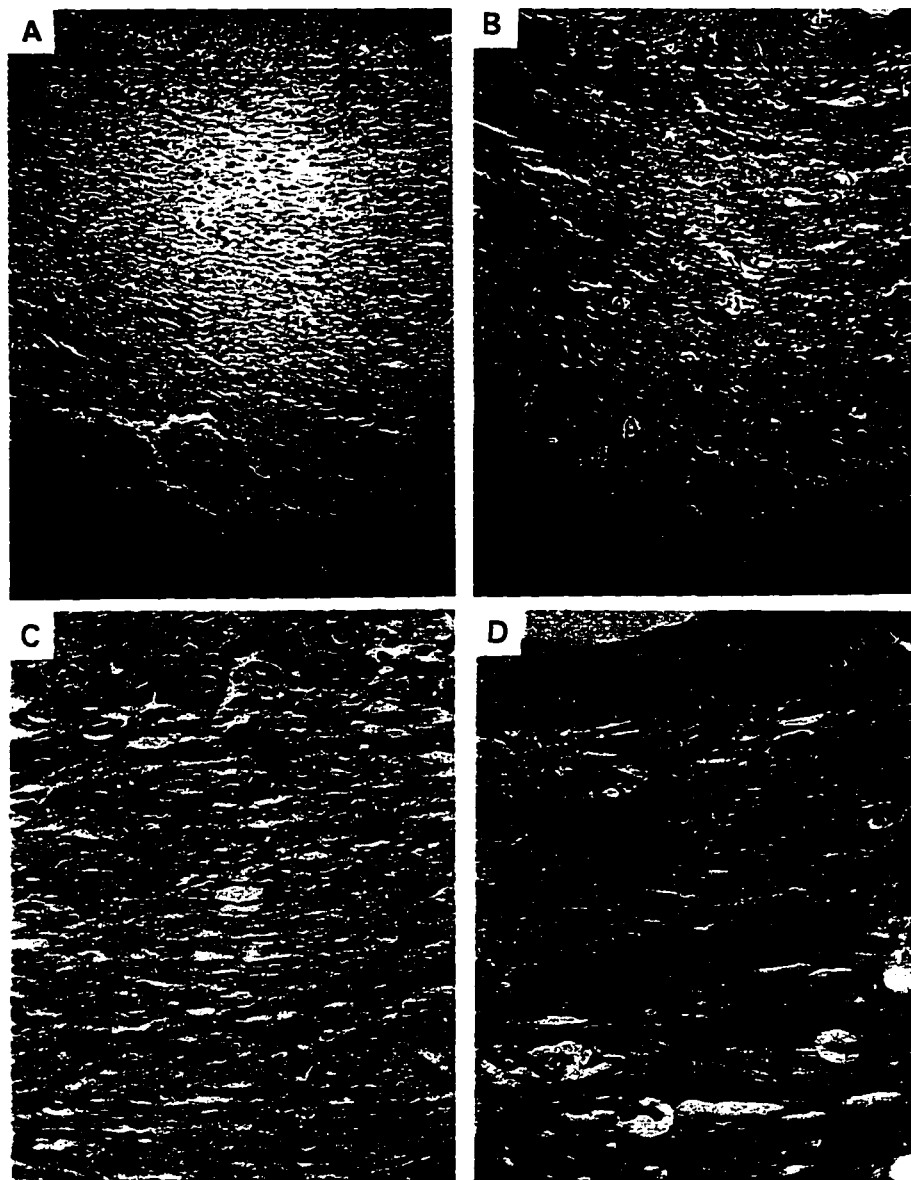
Figure 8   Decorin staining in the IFN-α2b treated wounds and control wounds 21 days after wounding.   Panel A shows a 50x magnification of the IFN-α2b treated wound at 21 days which was stained for decorin.   Weak staining can be seen throughout the entire central upper region of the wound which correlates with the region where α-SMA stains positively.   Panel B shows decorin staining of the control animal at 50x magnification which is intense throughout.   Panel C shows decorin staining of the IFN-α2b treated wound at 21 days at 100x magnification and panel D the wound of the control animal.
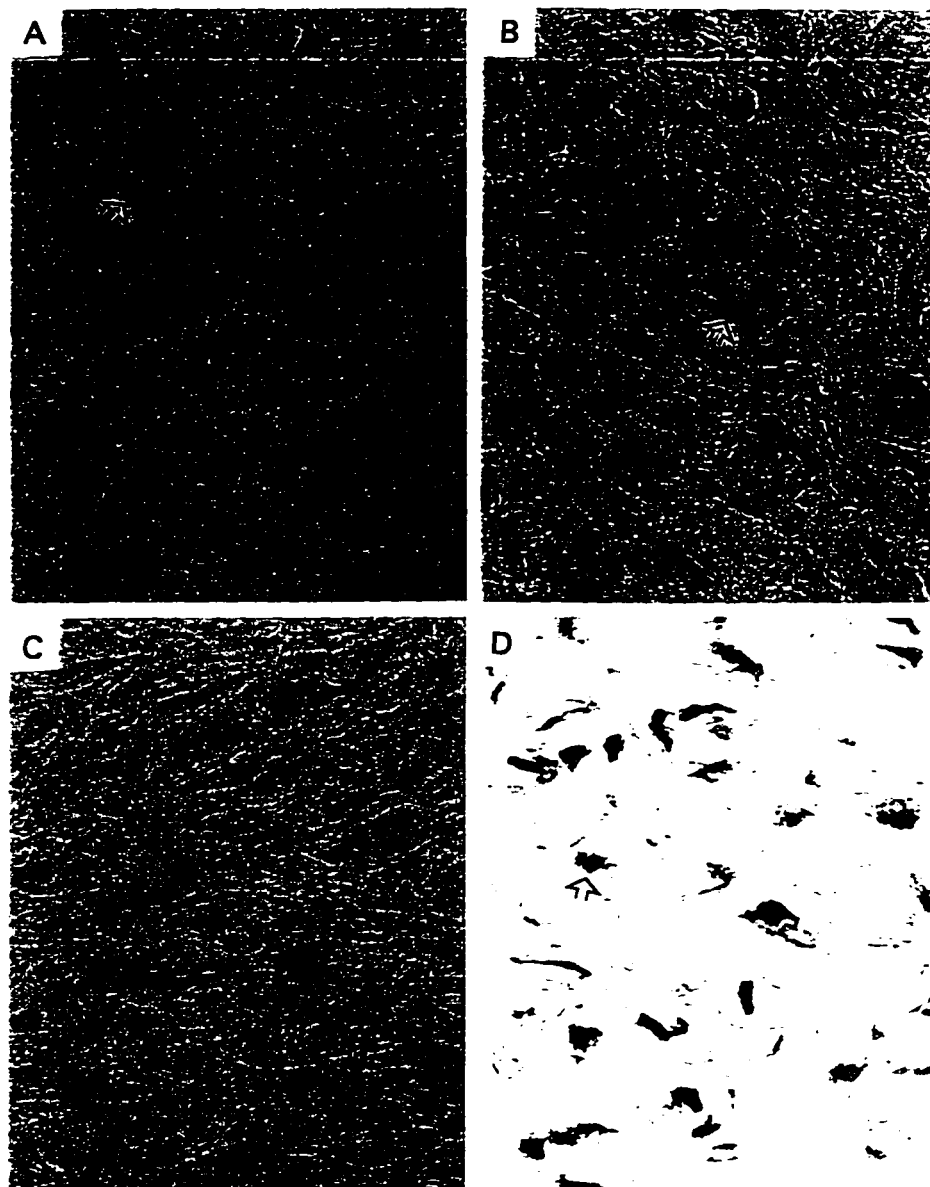
Figure 9 Apoptotic cells in the wounds. Panel A shows positively stained fibroblasts after performing the TUNEL assay on the IFN-α2b-treated wound sections 21 days following wounding. Panel B shows the wound from the control animal 21 days following wounding. Panel C is the control where the TdT was left out of the reaction buffer. Panel D is the positive control where the tissue sections were digested with DNAse prior to exposing them to the reaction buffer. Panel E is a graphic depiction of the quantitative analysis of the percent of apoptotic cells in the IFN-α2b-treated and control wounds 7, 14, and 21 days following wounding.

E

Number of days after wounding
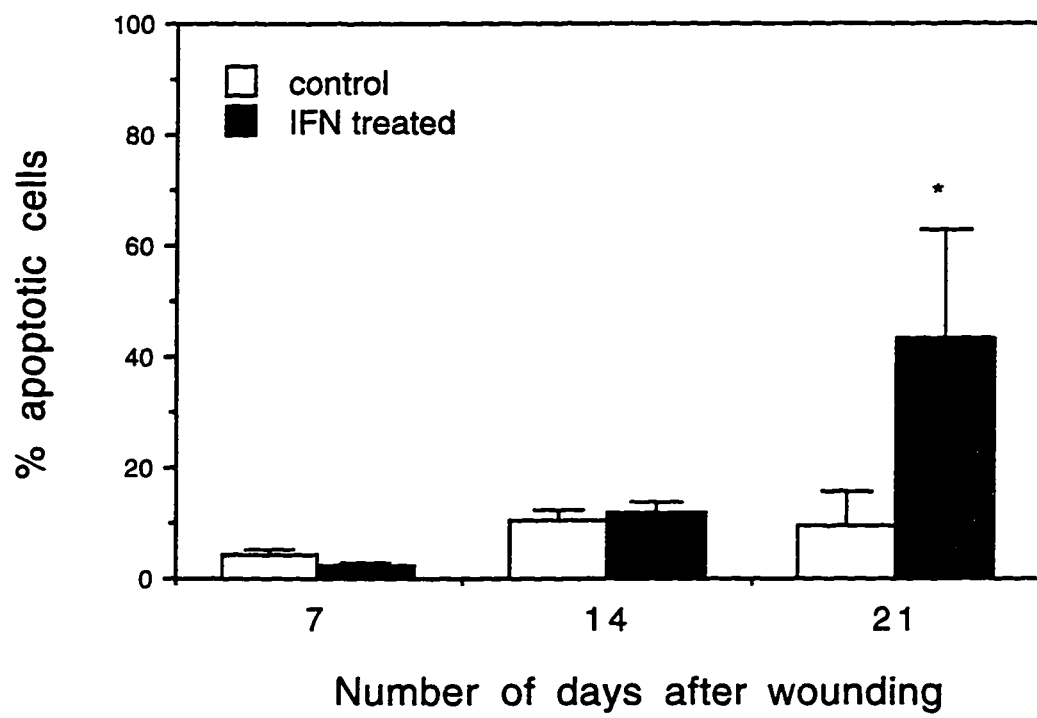
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CHAPTER 5:

MYOFIBROBLASTS AND APOPTOTIS IN HUMAN HYPERTROPHIC SCAR

AND NORMAL DERMIS AND THE EFFECT OF INTERFERON-α2b

5.1 ABSTRACT

The development of scar contracture and fibrosis following a deep partial or full thickness dermal injury correlates with the functional morbidity associated with these injuries. The myofibroblast was initially identified in granulation tissue as a specialized fibroblast which had contractile properties. This characteristic was attributed an increase in intracellular microfilament bundles similiar to those seen within smooth muscle cells. It was later shown that the myofibroblast expressed the α-smooth muscle actin (α-SMA) isoform which is characteristic of smooth muscle cells and since has come to define a myofibroblast. In this study the expression of α-SMA was quantitated in deep dermal wounds of burn patients that went on to develop hypertrophic scar (HSc) or healed with normal scar formation and was compared to normal wound healing in clean, moderately deep skin graft donor sites within the same patient. The percentage of fibroblasts that stained positive for α-SMA was shown to be significantly higher within HSc tissue relative to normal scar or normal tissue from the same patient. Staining for α-SMA was most intense in whorled regions where abnormal collagen fibrils were present yet in those areas where collagen fibres and fibre bundles were visible minimal staining was present. No correlation was apparent between the Vancouver Burn Scar Assessment (VBSA) ratings, as a measure of the clinical severity of HSc, and percentage of myofibroblasts present. The total number of fibroblasts in granulation tissue and HSc was found to be significantly greater than that of normal tissue

and decreases as the scar matures, which may significantly affect contraction. Tissue sections obtained from nine patients with severe HSc over greater than 5% total body surface area who had received systemic administration of IFN-α2b to promote remodelling showed a general reduction in myofibroblasts and total number of fibroblasts but this did not reach a level of statistical significance. The induction of apoptosis may contribute to the resolution of HSc over time as there was a significant increase in the percentage of apoptotic cells within HSc relative to normal dermis from the same patients. The administration of IFN-α2b may contribute to this increase since the percentage of apoptotic cells increased following the initiation of treatment.

5.2 INTRODUCTION

The development of HSc following a burn injury or any other major trauma to the skin is associated with contracture formation over joints or regions of mobile skin such as eyelids. It is the scar contractures that leads to the functional morbidity which results in delayed return to work and reintegration into society (14). The treatment of scar contractures includes conservative treatments such as splinting, pressure therapy, and serial casting (3, 24, 29); however, these are time consuming, uncomfortable and often unsuccessful. Surgical release may also be performed but the recurrence of contractures is common if the scarring process is still in an active phase (6).

It is generally agreed that scar contraction is a fibroblast based process. The myofibroblast was initially identified in granulation tissue as a specialized fibroblast which had contractile properties attributed to it based on morphologic features of both a fibroblast and a smooth muscle cell, including undulations of the nucleus, increased microfilament bundles within the cell and abundant endoplasmic reticulum (15). Since the development of a monoclonal antibody that specifically binds to the α-smooth muscle actin (α-SMA) isoform (34), the myofibroblast is usually defined as a fibroblast that stains positively for α-SMA.

The interferons (IFN) has been proposed as a potential therapeutic agents for the treatment of fibroproliferative disorders, based on its ability *in vitro* to decrease fibroblast proliferation (23), to down-regulate the production of collagen and fibronectin (19, 22, 37) and to enhance the production of collagenase (20). Reduction of the rate and extent of contraction *in vitro* has been shown using IFN-α, IFN-γ, and IFN-β (7, 27, 31). Although the effect of IFN on human scar contraction *in vivo* has not been measured directly, intralesional injections of IFN-α2b and IFN-γ led to a reduction in the mass of keloids, HSc and Dupuytren's disease nodules (4, 21, 28). Staining for α-SMA was reduced in intensity and distribution following 4 weeks of intralesional IFN-γ treatment with slight re-expression 12 weeks after treatment was discontinued (28). Previous research has correlated the disappearance of myofibroblasts in wound healing with apoptotic cell death (9, 11), although the mechanism of induction is not yet known. In addition, the more rapid disappearance of myofibroblasts in open wounds

covered with full thickness flaps compared to a split thickness flap, has been attributed to the accelerated induction of apoptosis (16). Conversely, a lack of induction of apoptosis may contribute to the excessive cellularity and contraction associated with HSc. One of the mechanisms by which IFN-α may be of therapeutic value in the treatment of fibroproliferative disorders is through the induction of apoptosis which has been previously demonstrated to be the effect of this cytokine in several cell lines, including activated T lymphocytes, squamous cell skin cancer cells and myeloma cells (8, 13, 30).

In order to evaluate the expression of α-SMA in wound healing that resulted in HSc formation and normal wound healing within the same individual, punch biopsies of granulation tissue were obtained from patients who had sustained partial or full thickness burn injuries, with the same location being re-biopsied several months later. As a normal wound healing control, biopsies of normal skin were taken from the donor sites prior to skin grafting procedures. The same site was rebiopsied several months later, as an example of a partial thickness wound that did not result in HSc formation. Biopsies obtained over a six month period from patients treated with IFN-α2b were also assessed to monitor the effect on α-SMA expression and the induction of apoptosis. Our results show that there are significantly more myofibroblasts within HSc than normal tissue, localized to regions where there are disorganized collagen fibrils rather than collagen fibre bundles. The number of myofibroblasts in normally healing wounds did not vary significantly from that in uninjured dermis. The overall percentage of

myofibroblasts was found to be double that in normal tissue and decreased in interferon treated patients as did the total fibroblast number, however the reduction did not reach a level of statistical significance. When evaluated as a function of time, the percentage of apoptotic cells in HSc relative to their normal control was low in early wound healing and significantly increased as a function of time postburn and following IFN-α2b treatment.

5.3 MATERIALS AND METHOD

Tissue biopsies

Patients recruited into this study had sustained thermal injuries that required surgical intervention and were treated at the University of Alberta Hospitals Firefighters' Burn Treatment Unit. Patients were recruited and tissues were sampled after informed consent had been obtained and according to the guidelines of the University of Alberta, Faculty of Medicine Research Ethics Board.

Skin biopsies were collected from two groups of patients. In the first group biopsies of granulation tissue, normal skin, healed burn wounds and healed donor sites were all obtained from the same patient. The granulation tissue and normal tissue was biopsied when the patient went to the operating room for skin grafting. The healed burn wound and healed donor site biopsies were obtained when the patient returned to the outpatient clinic for follow-up (Table I). The healed burn wound represented a partial thickness or full thickness wound that healed with or without HSc and the healed donor site

represented a control wound that healed without HSc formation (normal scar). A total of six patients or twenty four biopsies were studied.

In the second group, biopsies were obtained from nine patients who had developed HSc over greater than 5% of their total body surface area (TBSA) following a thermal injury and who had been recruited into a phase II clinical trial of interferon-alpha 2b (IFN-α2b) (INTRON-A®, Schering, NJ) (36). Hypertrophic scar and normal tissue biopsies (6 mm punch) were obtained prior to subcutaneous administration of 1×10^6 units per day for a week, then 2×10^6 units of IFN-α2b three times per week for 24 weeks. Further biopsies of HSc were obtained every other month and one month after the conclusion of therapy. A second biopsy of normal tissue was taken at the termination of IFN-α2b treatment (36). All biopsies were immediately placed in 4% paraformaldehyde. Assessment of the severity of HSc was made using the Vancouver Burn Scar Assessment (35).

Materials

Biotin-16-2'-deoxyuridine-5'-triphosphate, proteinase K, dUTP, 4-nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate and terminal deoxynucleotidyl transferase (TdT) were obtained from Boehringer Mannheim (Indianapolis, IN). RNase-free DNase was obtained from Promega (Madison, MI). Casein blocking buffer was obtained from Cambridge Research Biochemicals (Northwich, Cheshire). Anti-alpha smooth muscle actin (clone 1A4),

bovine serum albumin (BSA), 3, 3'-diaminobenzidine (DAB), 3-aminopropyl-triethoxy-silane (Aptex), monoclonal peroxidase-anti-peroxidase soluble complex antibodies, ExtrAvidin®-Alkaline Phosphatase and normal goat serum were obtained from Sigma Chemical Company (St Louis, MO). The bridging goat F(ab')2 fragment to mouse IgG (whole molecule) was obtained from ICN Pharmaceuticals Inc (Aurora, Ohio).

Immunohistochemistry

The tissue biopsies which had been fixed in 4% paraformaldehyde were embedded in paraffin and cut into 4-5 μm sections which were placed on Aptex coated slides. All sections were heated to 60° C for 10 min then dewaxed in xylene and rehydrated. Endogenous peroxides were inactivated by treating all sections with 10% H_2O_2 in methanol for 6 min. After washing with PBS, sections were incubated for 30 min at room temperature in blocking agent (10% v/v normal goat serum, 5% bovine serum albumin w/v in PBS) and then incubated overnight at 4° C with the appropriate antibody. The α-SMA antibody was diluted in PBS/Tween 0.05% at a 1:100 ratio. The controls were incubated with plasmacytoma cell culture supernatant diluted in PBS/Tween 0.05% at a 1:10 ratio. After warming to room temperature the sections were incubated with goat F(ab')2 fragment to mouse IgG (whole molecule; 1:50 dilution in PBS/Tween 0.05%) for 1.5 hours, washed with PBS, then incubated with monoclonal peroxidase-anti-peroxidase soluble complex at a 1:200 dilution for 1.5 hours and

washed with PBS. The colorimetric reagent was hydrogen peroxide/DAB and all sections were counterstained with haematoxylin.

Counting of α-SMA positive fibroblasts was performed on 10 randomly selected high power fields per section. The total number of fibroblasts was also counted on the same section thus allowing calculation of the percentage of myofibroblasts (See Appendix I). Smooth muscle cells associated with blood vessels and erector pili were excluded from the α-SMA positive fibroblast count and the total fibroblast count.

TUNEL Assay

The *in situ* demonstration of DNA fragmentation was performed using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) as previously described (17). All sections were treated as described for immunohistochemistry up to the point of rehydration. After washing with ddH2O, the sections were incubated for 20 min at 37° C in 2 x sodium chloride and sodium citrate (SSC) (pH 7.0) then washed several times in ddH2O. All sections were bathed for 5 min at room temperature (RT) in 10 mM Tris-HCl (pH 8.0) then digested for 15 min at 37° C with proteinase K (20 μg/ml in 10 mM Tris-HCl). After washing several times in ddH2O the tissue sections were fixed for 4 min in 4% paraformaldehyde then washed several times with ddH2O. The positive control was incubated in DNase buffer for 5 min at RT then digested with DNase (1 μg/ml) at 37° C for 30 min. The positive

control was washed with ddH$_2$O then all sections were incubated for 5 min at RT in TdT buffer (pH 7.2). All sections were incubated in reaction buffer (94.5 µl ddH$_2$O, 7.0 µl TdT buffer, 4.4 µl Bio-16-dUTP, 4.4 µl dUTP and 1.5 µl TdT) for 1 hour at 37° C in a humidified chamber, except the negative control where the TdT was omitted. To terminate the reaction the sections were transferred to 2 x SSC for 15 min at RT. All sections were washed with PBS then incubated for 15 min at RT in blocking agent (1:5 casein blocking buffer, 10% v/v normal goat serum, and 5% bovine serum albumin w/v in PBS). The sections were then incubated at RT for 30 min in Extra-Avidin phosphatase (1:250) in PBS/Tween 0.5%. The sections were washed with PBS then incubated with colorimetric buffer (0.1 M Tris, 0.1 M NaCl, 0.25 M MgCl$_2$; pH 9.5) for 5 min at RT. The colorimetric reagent was 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

The percentage of positive cells was calculated by counting the number of positive cells in 10 randomly selected fields and comparing it to the total number of cells within a serial section stained with haematoxylin (See Appendix I). Cells that were obviously endothelial cells or epithelial cells such as in vessels, hair follicles, sweat glands, sebaceous glands and the outer epithelial layer were eliminated from both positive cell counts and total cells counts.

Statistics

Student's paired t test two-tailed was used for all comparisons. Results are reported using p value, mean and standard error of the mean. Linear regression analysis was done using Instat software (GraphPad Software, Inc., San Diego, Cal).

5.4 RESULTS

In HSc tissues, the localization of the α-SMA positive fibroblasts tended to be in more densely populated cell regions, but was not exclusive to any particular region of HSc (Figure 1); however fewer α-SMA positive fibroblasts were generally visible in regions where more organized collagen fibres were present. Positive staining was consistently seen in the vessels where α-SMA is characteristically expressed and in the epidermal cells where it is considered to be artifactual.

As indicated in Table I, four of the six patients from the first group developed HSc in their burn wound site and all of the patients in the second group had already developed HSc as one of the criteria for inclusion in the study. The definition of clinically significant HSc was arbitrarily set at a VBSA rating of greater than 3, as this would indicate deviation from normal on three of the four parameters. Although not indicated in Table I, none of the patients from the first group developed HSc at their donor site. The number of myofibroblasts was calculated as the percentage of α-SMA positive staining fibroblasts per section. Ten randomly selected

high powered fields were measured in each section. The percentage of myofibroblasts within the HSc sections ranged from 1% to 65%, with a mean of 23.2%±5.2. All of the patients that had more than 35% positive fibroblasts had sustained burn injuries to greater than 70% of the total burn surface area (TBSA). The patients with burn wounds less than 60% TBSA all had fewer than 30% α-SMA positive fibroblasts. When comparisons were made between the HSc and normal tissue biopsy of those patients that developed clinically significant HSc there was a statistically significant difference (23.2%±5.2 vs 6.2%±1.4; n=13) (p<0.005) (Figure 2). The number of α-SMA positive fibroblasts in the two patients who did not develop HSc of their burn wound was in the lower-range (1% and 13%). The mean percentage of α-SMA positive fibroblasts in the donor site wounds which healed normally was 5.8%±2.9 (n=6). Comparisons between the normal dermis and healed donor sites in the first group of patients revealed no significant difference.

It is our hypothesis that as the severity of HSc increases the presence of myofibroblasts will increase. In order to correlate the presence of myofibroblasts with the extent of HSc development, the Vancouver Burn Scar Assessment (VBSA) was performed at the same time as the HSc biopsies were obtained. Using least squares linear regression analysis for the comparison of the percentage of myofibroblasts and the VBSA it was found that there was no significant correlation (p value=0.4255, r^2=0.049; n=15) (Figure 3).

In the fibroblast-populated collagen lattice model assay (2) it has been shown that the rate and extent of contraction correlate with the number of cells. One of the factors that may contribute to

scar contraction and fibrosis is an increase in total cell number relative to that of normal skin. The total number of fibroblasts per high power field varied significantly between biopsies from normal dermis and granulation tissue (4.7±0.5 vs 12.0±2.6; p<0.001) and between normal dermis and HSc (4.7±0.5 vs 10.5±1.0; p<0.05) but not between normal dermis and normal scar (4.7±0.5 vs 6.8±0.9) (Figure 4A). When the total number of fibroblasts in HSc was compared as a function of time following burn injury there was a gradual reduction between early (0-4 months postburn), mid (5-18 months) and late (19-30 months) HSc (Figure 4B). The reduction between early and late is statistically significant at a p value of less than 0.01.

The results of a phase II clinical trial had suggested that IFN-α2b may be of therapeutic value in the treatment of patients who develop HSc (36). Comparison of the percentage of myofibroblasts in the HSc at the time of the initial biopsy relative to the end of the interferon trial showed a statistically significant reduction in three of the nine patients but overall this was not statistically significant (26.9%±6.6 vs 16.0%±2.8) (Figure 5). Another possible mechanism by which interferon may exert an effect is through a reduction in the total number of fibroblasts. Comparisons of the average number of fibroblasts in ten randomly selected fields were made between tissue sections of HSc obtained prior to IFN-α2b treatment and sections from adjacent HSc at the completion of the trial. Although three of the nine patients showed a statistically significant reduction in the total number of fibroblasts the overall reduction was not statistically significant (9.8±1.4 vs 7.7±1.3) (Figure 6).

Although the reduction in cell number did not reach a level of statistical significance, the loss of cellularity may be clinically and biologically important. It has been suggested that the induction of apoptosis may mediate the decrease in cellularity when granulation tissue evolves into a scar in normal wound healing (11) therefore apoptotic cell death was investigated as a possible mechanism by which IFN-α2b may facilitate the maturation of HSc. When the percentages of apoptotic cells from all the HSc biopsies were compared to their normal tissue counterparts the level was reduced in early (0-4 months postburn) HSc, was similiar to normal in the mid range (5-18 months) and significantly higher in late (19-30 months) HSc (Figure 7A). Although it has been previously reported that apoptotic cells in normal rat skin were generally not apparent (11) positive cells were consistently present in normal dermis of human burn survivors. Quantitative analysis revealed that the percentage of apoptotic positive cells relative to normal in early (75.1%±25.7; n=5) HSc differed significantly from that of mid (110.3%±15.8; n=24) and late HSc (220.6%±31.2; n=19)(Figure 7B).

To attempt to establish whether this was an IFN-α2 b treatment-dependent induction of apoptosis, the pretreatment biopsy, the biopsy which had the highest percent of apoptotic cells following IFN-α2b treatment and their final biopsy following treatment were compared to the normal tissue biopsy (Figure 8). Although their pretreatment biopsy did not differ significantly from normal (33.4%±6.5 vs 20.9%±4.5; n=9), the percentage of apoptotic cells following administration of IFN-α2b was significantly increased compared to normal (43.5%±5.6 vs 20.9%±4.5; n=9; p<0.01).

The final biopsy following IFN-α2b treatment also differed significantly from normal although to a lesser extent (26.3%±4.7 vs 20.9%±4.5; n=9; p<0.05).

5.5 DISCUSSION

It is well established that scar contractures are associated with the development of HSc following partial thickness or full thickness dermal injuries and those wounds that take longer to heal are more likely to develop HSc (10). Our results suggest that burn survivors who develop HSc have an increased number of myofibroblasts and particularly those patients who have sustained massive burn injuries. Comparisons within the same patient of normally healing wounds following partial thickness dermal injuries (donor sites) shows that this is specific to sites that progress to pathologic wound healing and is not a generalized response. Although a few fibroblasts within normal tissue stained positive for α-SMA the number was generally low.

As has been previously reported, HSc is characterized by the presence of nodules of thin collagen fibrils (5, 25). Fibroblasts stained for α-SMA were generally concentrated in the regions of the scar that contained poorly organized collagen fibrils and a high density of cells. It has been previously reported that α-SMA staining in HSc was restricted to the nodules (12). Our findings confirmed that staining was seen within the nodular regions but it was not uncommon to see it in the apparently more mechanically stressed whorls that surrounded the nodules. In both situations however

there were thin collagen fibrils surrounding the myofibroblasts. In those regions where more organized collagen fibre bundles were observed, often within the same tissue section, fewer α-SMA stained fibroblasts were observed, which suggests that the myofibroblast phenotype may be regulated by the organization of the extracellular matrix.

The clinical assessment scale that is most commonly used to quantitate the severity of HSc is the Vancouver Burn Scar Assessment (35). If the assumption is made that there is an increased number of myofibroblasts with worsening HSc it would be expected that the number of α-SMA positive fibroblasts would correlate with a higher VBSA rating, however this is not the case. Two interpretations of these data are possible. First, that the presence of myofibroblasts does not correlate with the severity of HSc and/or second, although the reliability of the VBSA has been tested (1, 35) no measurement of its validity has been conducted. Hence it may not accurately reflect the severity of HSc.

Fibroblast proliferation is required for wound healing to occur (26). As the granulation tissue evolves into scar tissue there is a normalization of cell number. In HSc one of the major contributing factors to development of scar contractures and fibrosis may simply be an increased number of cells. Within the fibroblast-populated collagen lattice model, the rate and extent of contraction is comparable between normal fibroblasts and HSc fibroblasts (27). If the number of cells within the lattice is increased there is a corresponding increase in the rate and extent of contraction (2). A similiar situation may be occurring within HSc, where twice as

many fibroblasts are present compared to normal tissue. That, combined with the altered rate of collagen production (18) and disorganized arrangement (25) and altered proteoglycan profile (32, 33), may cause scar contraction without the need for a specialized cell type.

Systemic administration of IFN-α2b appears to improve the quality of healing resulting in a more supple, pliable tissue (36). Following the administration of IFN-α2b there appeared to be a reduction in the number of α-SMA positive fibroblasts and total fibroblast number but this did not reach a level of significance. One explanation for these findings may simply be a reflection of the *in vitro* observation that interferon reduces the rate of fibroblast proliferation (23) thereby normalizing cell number. Additionally, it has previously been demonstrated that there is a correlation between the expression of α-SMA in fibroblasts and apoptosis (11). The reduction in α-SMA positive fibroblasts and the total number of fibroblasts in sections obtained from patients treated with IFN-α2b may be a result of the induction of apoptosis. The significant increase in the percentage of apoptotic cells with time relative to their normal tissue controls suggests that apoptosis is involved in the conversion of HSc tissue into mature scar. The direct effect of IFN-α2b in this conversion is difficult to assess independent of time but the increase in the percent of apoptotic cells compared to normal biopsies following interferon treatment suggests that this is a contributing factor in the clinical improvement seen. Examination of tissue biopsies obtained in a double blind trial would further

assist in the conclusive determination of whether IFN-α2b decreases the myofibroblast population and induces apoptosis.

5.6 Figures

Table 1   Patient demographic information

Patient	TBSA	Age	Sex	VBSA	Days from burn to biopsy (normal/ granulation) (Days)	Months post burn to biopsy (donor/scar) (Months)
Group 1						
1	5	67	M	0	5	2
2	18	35	M	1	7	12
3	85	28	M	12	75	12
4	3	42	M	6	6	3
5	25	49	M	6	4	4
6	40	41	M	9	17	3
Group 2						
7	32	49	M	12		17
8	70	26	M	6		20
9	30	49	M	13		17
10	65	36	M	10		4
11	60	31	M	8		20
12	45	25	M	9		6
13	20	46	F	12		16
14	40	10	F	11		12
15	85	29	M	7		8
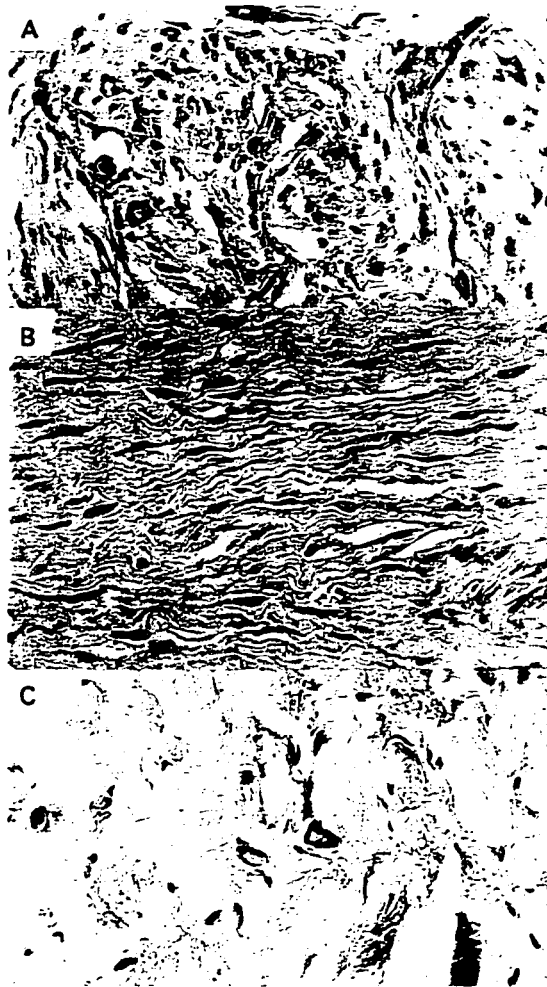
Figure 1   Localization of α-smooth muscle actin positive fibroblasts within HSc tissue.   Positive staining of fibroblasts for α-SMA was localized to regions of disorganized collagen fibrils and to a lesser extent where collagen fibres and fibre bundles were present.   Panel A, B, and C are all from the same patient biopsy but different regions of the tissue, all stained for α-SMA.   Panel A demonstrates intense fibroblast staining in the centre of a nodular region where there are disorganized collagen fibrils and capillaries on the outer edge of the nodule.   Panel B demonstrates a region where the α-SMA positive fibroblasts are arranged in parallel alignment, with the collagen fibrils aligned in a waved pattern but parallel to the fibroblasts.   Panel C demonstrates a region where more normal collagen fibres and fibre bundles are visible.   Although the vessels at the centre of the panel are intensely stained there are few stained fibroblasts and the tissue is markedly less densely populated. (100x magnification)
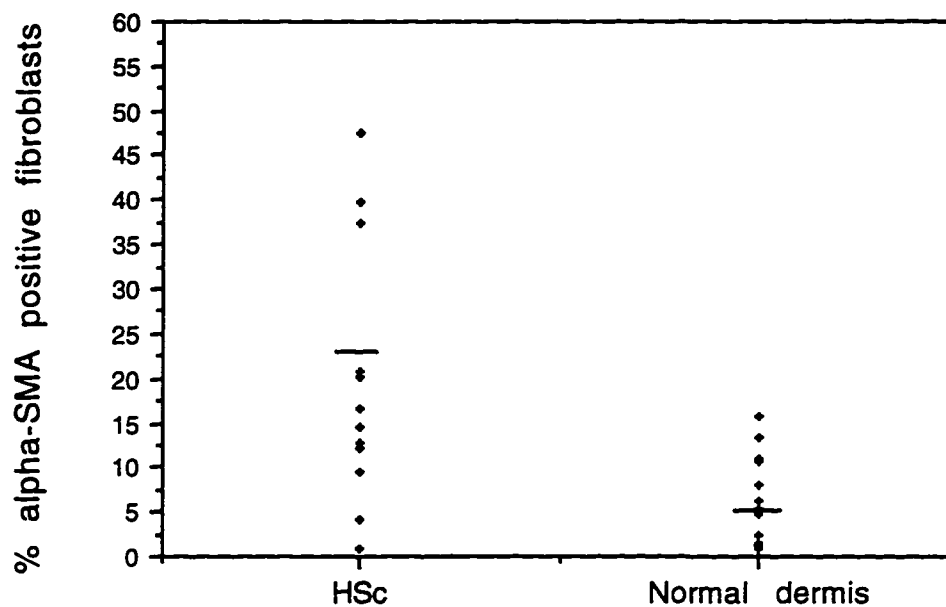
Figure 2 Percentage of α-smooth muscle actin positive fibroblasts in hypertrophic scar relative to normal dermis. Thirteen biopsies of hypertrophic scar and normal dermis from the same patient are shown where 10 random high power fields were quantitated per biopsy. The bars indicate the mean for each group (23.2%±5.2 vs 6.2%±1.4; n=13)(p<0.005).
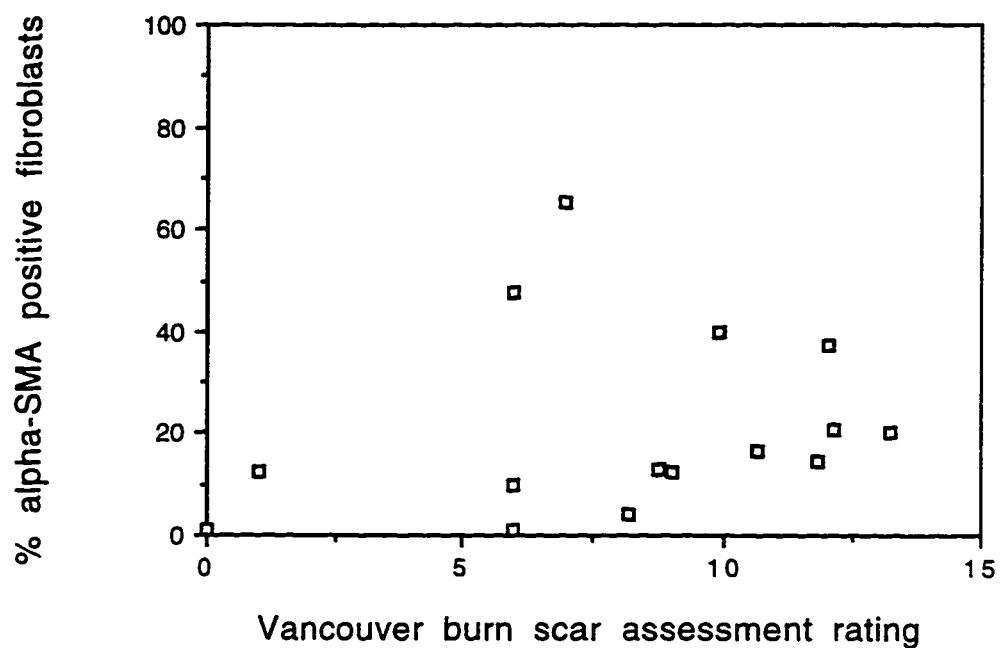
Figure 3   Percentage of α-smooth muscle actin positive fibroblasts does not correlate with clinical severity of HSc.   Comparison of the percentage of α-smooth muscle actin positive fibroblasts and ratings on the Vancouver Burn Scar assessment showed that there was no correlation ($p=0.4255$; $r^2=0.049$; $n=15$).
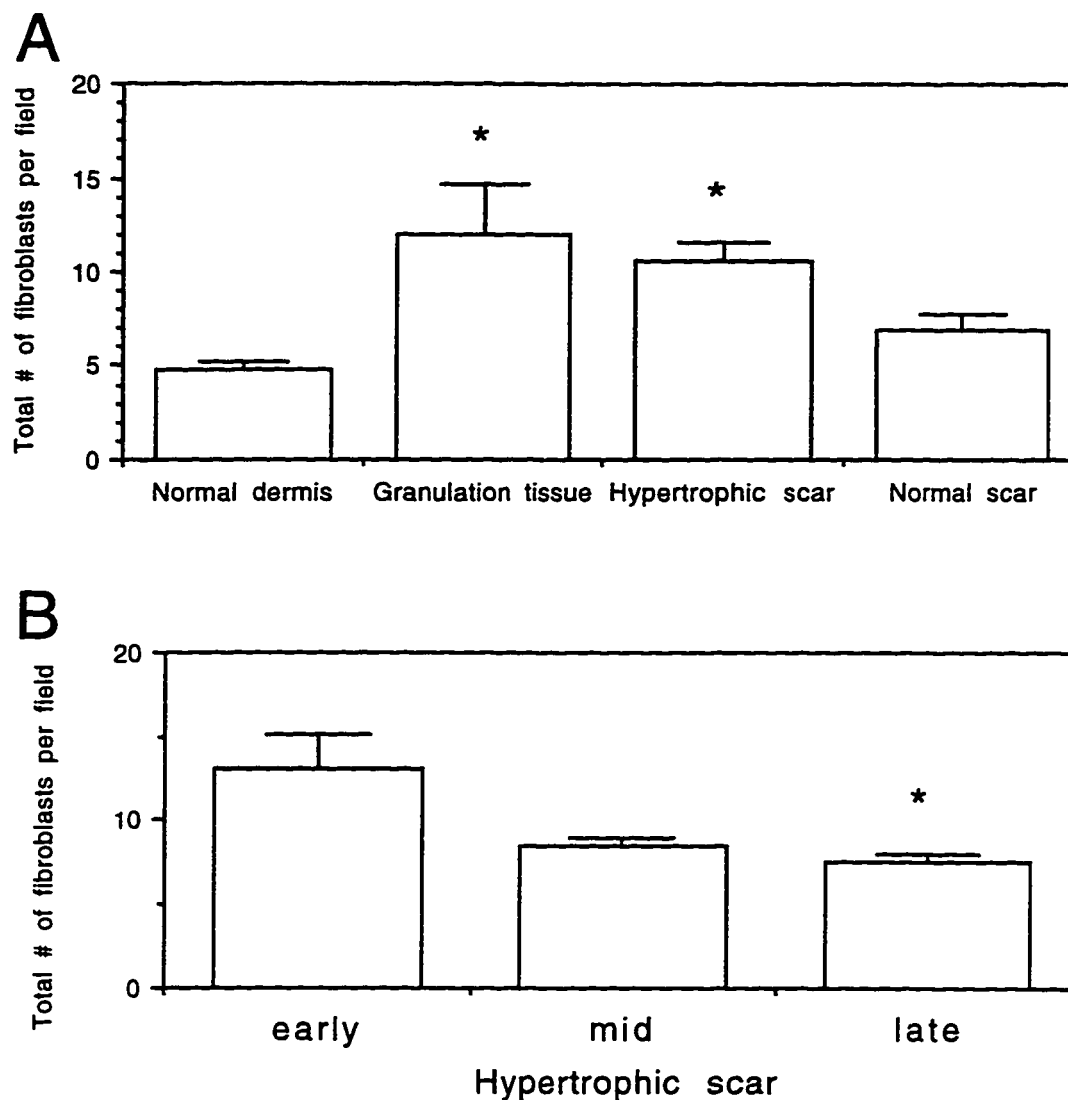
Figure 4   The total number of fibroblasts per high power field differs between various types of wound healing and returns to a more normal level as the scar matures.   The total number of fibroblasts per high power field was averaged from 10 randomly sampled fields.   Panel A depicts the difference between the total number of fibroblasts in granulation tissue and HSc relative to normal dermis (12.0±2.6 and 10.5±1.0 vs 4.78±0.5).   Normal scar did not differ significantly from normal dermis (6.8±0.9).   Panel B depicts the gradual reduction in total number of fibroblasts as a function of time, where early scar is 0-4 months postburn (13.1±2.0) (n=5), mid is 5-18 months (8.5±0.4) (n=25), and late is 19-30 months (7.6±0.5) (n=20).
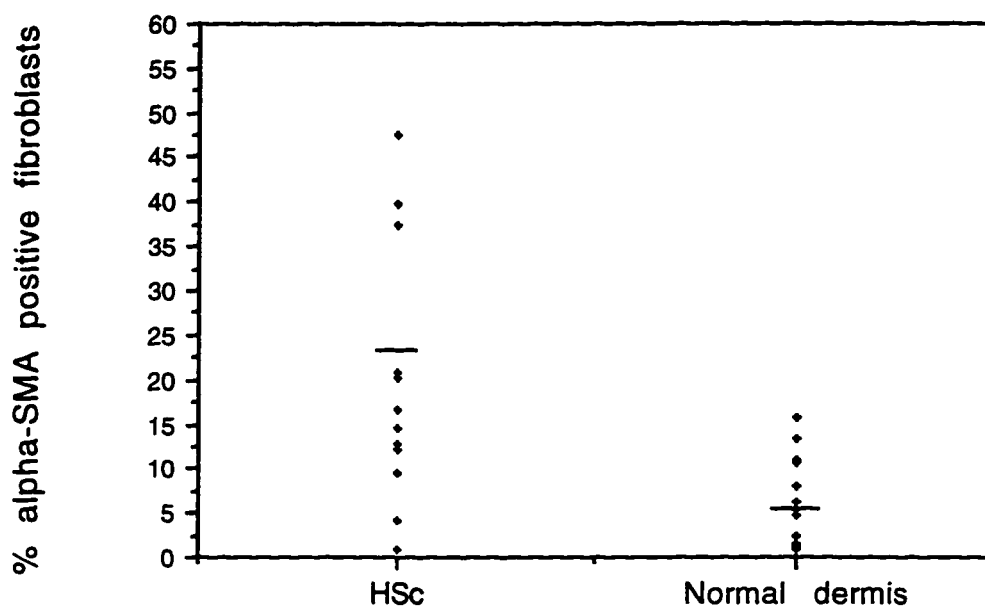
Figure 5   The percentage of α-smooth muscle actin positive fibroblasts decreases following IFN-α2b treatment. The number of α-smooth muscle actin positive fibroblasts was compared in HSc tissue sections from the same patient prior to and six months following systemic administration of IFN-α2b. Individual patient comparisons demonstrated a significant reduction in the percentage of positive cells in three of the nine patients however the overall reduction did not reach a level of significance (26.87%±6.57 vs 15.99%±2.84) (n=9).
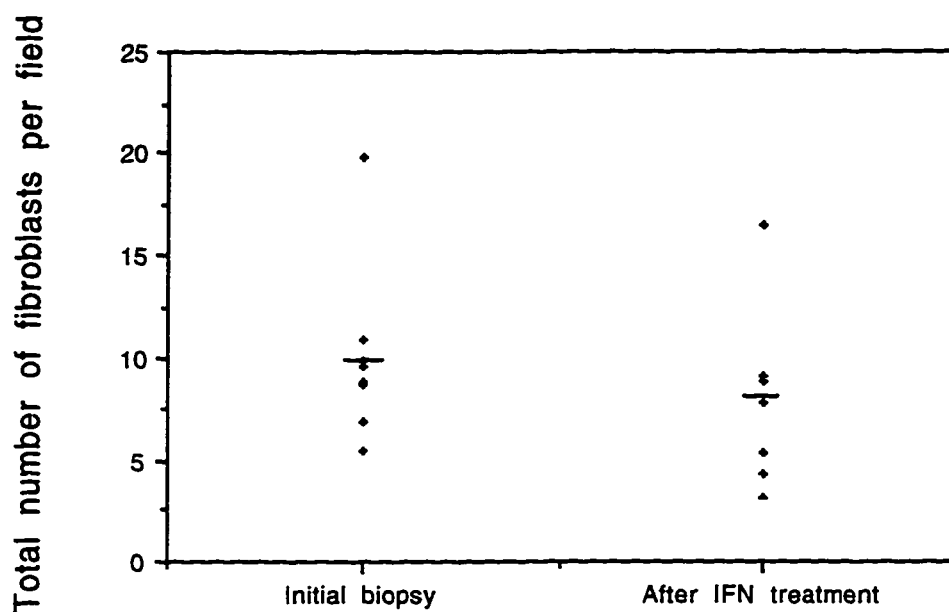
Figure 6   The total number of fibroblasts per high power field of hypertrophic scar tissue decreased following IFN-α2b treatment. The total number of fibroblasts in HSc tissue sections from the same patient prior to and six months following systemic administration of IFN-α2b showed a significant reduction in three of the nine patients.   Although overall comparisons do not show a statistically significant reduction there appears to be a general downward trend (9.8±1.4 vs 7.7±1.3) (n=9).
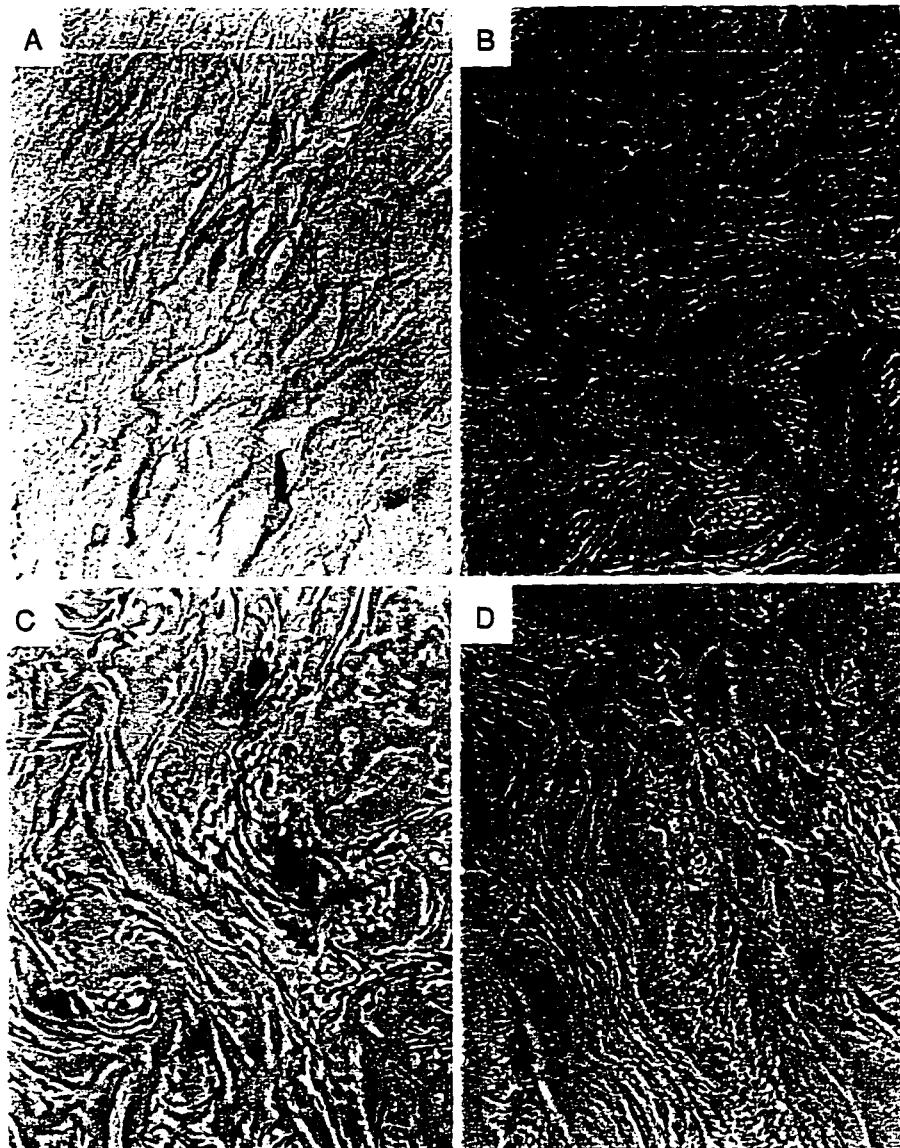
Figure 7A   Representative examples of apoptotic cells within hypertrophic scar at various times following burn injury relative to their normal dermis. The TUNEL assay identifies apoptotic cells following colorimetric development as blue cells.   Panel A: An example of HSc in the early period following a burn injury (0-3 months).   Panel B: HSc 19 months or later following a burn injury. There are significantly more positive cells than in Panel A.   Panel C is an example of normal dermis which has more positive cells than the early HSc in Panel A but that seen in the late HSc in Panel B. Panel D shows the control tissue section where terminal transferase was omitted from the reaction. (250x magnification)
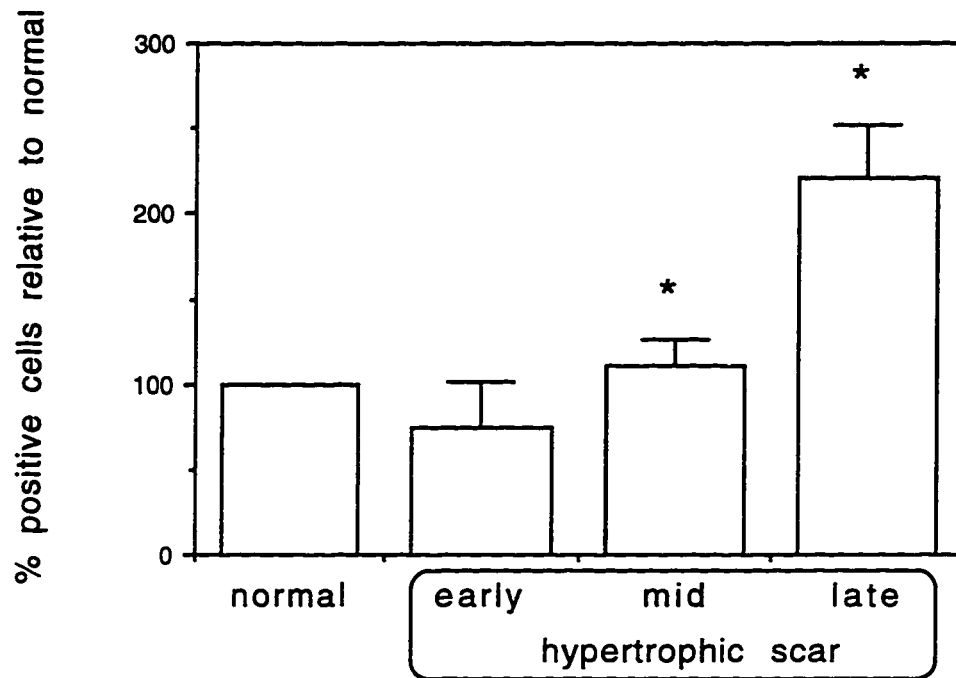
Figure 7B   The percentage of apoptotic cells in HSc increases as a function of time following burn injury relative to patient matched normal controls.   The percentage of apoptotic positive cells when compared to normal is reduced in early (0-4 months postburn injury) HSc (75.1%±25.7; n=5) although not to a level of statistical significance, similiar to normal in the middle time period (5-18 months) (110.3%±15.8; n=24) but statistically elevated relative to early HSc and was further elevated in the late time period (19-30 months) (221%±31; n=19) (*=p<0.01).
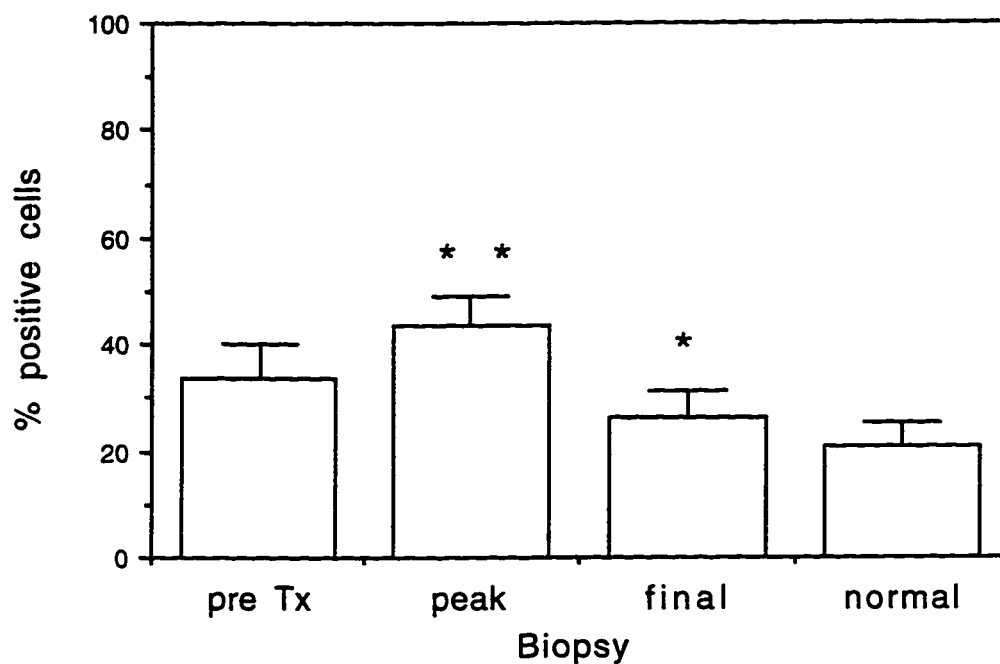
Figure 8   The percentage of apoptotic cells increased significantly in interferon treated patients relative to normal dermis. Pretreatment HSc biopsies did not significantly differ from that of normal values (33.4%±6.5 vs 20.9%±4.5) (n=9). The percentage of apoptotic cells following the administration of IFN-α2b was significantly increased relative to normal (43.5%±5.6 vs 20.9%±4.5) (n=9). The final biopsy also differed significantly from normal (26.3%±4.7 vs 20.9%±4.5) (n=9) (**p<0.01; *p<0.05).

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CHAPTER 6:

GENERAL DISCUSSION AND CONCLUSIONS

6.1 Discussion and Conclusions

Wound contraction and the development of scar contractures are complex, multifaceted processes where the production and functional coordination of cytoskeletal proteins imparts forces on and receives signals from the extracellular matrix proteins and soluble molecules via cell associated receptors and integrins (3, 15, 24). Wound contraction, particularly in animals (14), has positive consequences in that it accelerates wound closure, reduces the surface area and hence the potential for overwhelming microbial contamination, thereby enhancing the probability of survival. Conversely, scar contractures, which are common sequelae associated with deep partial or full thickness dermal wound healing in humans, contribute to negative outcomes including restricted function and poor cosmetic results. Rigorous comparisons of how these two processes differ is frustrated by the lack of an animal model which produces pathologic fibroproliferative scar similar to hypertrophic scar (HSc). This thesis represents an attempt to examine the potential therapeutic usefulness of interferon-$\alpha 2$ b (IFN-$\alpha 2$b) to inhibit or reduce the development of scar contractures in humans by utilizing an *in vitro* model system which simulates wound contraction, an *in vivo* wound contraction model and *in situ* analysis of human tissue sections.

The fibroblast-populated collagen lattice is an *in vitro* model system which simulates wound contraction (1). The first series of experiments were based on this model to allow for a more controlled examination of the effect of IFN-$\alpha 2$b. Using matched pairs of human

HSc and normal dermal fibroblast cultures established from patients recovering from a thermal injury, IFN-α2b exposure prior to lattice formation was found to significantly inhibit contraction in a treatment time-dependent manner. The pattern of lattice contraction was found to be triphasic as previously reported (13) and sensitive to serum which probably supplies transforming growth factor-ß (2) and platelet-derived growth factor (11). Comparison of HSc and normal dermal fibroblasts revealed no significant differences in ability to induce lattice contraction however contrary evidence has been presented (16, 17) suggesting that this finding may require further examination. Factors such as collagen concentration and cell number can significantly alter the rate and extent of lattice contraction (1), hence modifications in the experimental technique may enhance the sensitivity of the assay to detect differences in fibroblast strains ability to produce contraction. The use of normal dermal fibroblasts from the same patient as a control may also minimize the inter-subject variability. Northern blot analysis of mRNAs for the intracellular contractile proteins revealed that IFN-α2b significantly down-regulated mRNA levels for the actin isoforms ß and γ but had no significant effect on α-tubulin, vimentin and α-actinin which suggests that its effect may be through a reduction in actin production. The lattices were stained with rhodamine-labeled phalloidin to allow for visualization of the organization of filamentous actin. Marked morphologic alterations of the microfilament organization were associated with reductions in lattice contraction following IFN-α2b treatment. Two hours after lattice polymerization, actin filaments were seen to

extend three dimensionally in a star-like or arborized pattern in both the IFN-α2b-treated and untreated cells. However, after twenty four hours within the gel, marked variations were seen. As has been previously reported (23), the untreated fibroblasts became oriented in a bipolar-spindle or elongated shape as the microfilaments consolidated towards the apices of the cell. IFN-α2b-treated fibroblasts were less elongated and lacked the bipolarity and unidirectional orientation. The microfilaments appeared to be spread throughout the cytoplasm in a sheet, with broad focal adhesions as compared to the more focused terminations seen in the untreated cells.

The results of the *in vitro* studies support the therapeutic use of IFN-α2b however the *in vivo* environment is far more complex. In order to verify that IFN-α2b had a similiar effect *in vivo*, contraction of full thickness wounds was assessed in a guinea pig model. Osmotic pumps loaded with either IFN-α2b or sterile saline were implanted intraperitoneally. Seven days following implantation six full thickness punch biopsy wounds were created on the dorsum of each animal and monitored daily. Comparison between the IFN-α2b treated animal and saline control animals indicated that there was a significant reduction in the rate of wound contraction in the interferon treated animals. Tissue samples were collected from the guinea pigs prior to wounding and at weekly intervals over three weeks. Western blot analysis of the tissue indicated that although the relative amount of vimentin, as an indicator of mesenchymal cell number, was increased after wounding, the myofibroblast-associated cytoskeletal proteins α-smooth muscle actin (α-SMA) and

smooth muscle myosin were not. Interestingly although it would have been predicted that α-SMA would be expressed most intensely in early granulation tissue since that is the time period where contraction is most rapid, immunohistochemistry localized the expression of α-SMA within the central region of the wound 21 days after wounding only in the IFN-α2b treated animal. Staining for decorin showed a specific reduction in the region that correlated to the presence of α-SMA staining. The collagen organization associated with this region consisted of collagen fibrils as opposed to the fibres and fibre bundles seen in normal tissue or mature scar (12). This reciprocal relationship lends support to the suggestion that the presence of decorin facilitates the transition of hypertrophic scar into the more organized matrix (19, 20) and that "outside in" signaling (3) may significantly effect the expression of α-SMA. The increase in α-SMA staining in the IFN-α2b treated animals was correlated in time with an increased number of apoptotic cells but the localization did not strongly correlate. Taken together these findings suggested that the inhibitory effect of IFN-α2b was not through a reduction in the myofibroblast population but may be through the induction of apoptosis and an altered extracellular matrix protein profile.

Experimentation in humans is difficult since a minimal amount of manipulation is considered ethical and control of variables is difficult. In an attempt to extend the findings of the *in vitro* model system and the *in vivo* animal model to humans, a series of tissue samples obtained from human burn survivors were examined. The expression of α-SMA was quantitated in burn wounds that went on to

develop HSc and in those that healed with normal scar formation. These tissue sections were also compared to normal wound healing in the same patient where the donor site which developed normal scar was evaluated. Tissue sections obtained from patients who had been enrolled in a limited phase II trial of systemic IFN-α2b were also examined in an attempt to assess the effect of IFN-α2b on the expression of α-SMA as an indicator of contractile fibroblasts. The percent of fibroblasts that stained positive for α-SMA, or percent of myofibroblasts was shown to be significantly higher within HSc tissue relative to normal scar or normal tissue from the same patient. Staining for α-SMA was most intense in regions where abnormal collagen fibrils were present and in those areas where collagen fibres were visible, minimal staining was present. To assess whether the presence of myofibroblasts was indicative of the severity of HSc, the Vancouver Burn Scar Assessment ratings (21) were compared with the percentage of myofibroblasts present in tissue sections but there was no correlation. Unfortunately this rating scale does not directly measure scar contraction, nor would that have been feasible in this patient population as the conservative treatments employed to minimize scar contraction could not be controlled. The total number of fibroblasts in HSc tissue was found to be twice that of normal tissue which may significantly effect contraction. Tissue sections obtained from the patients who had received systemic administration of IFN-α2 b showed a general reduction in myofibroblasts and total number of fibroblasts but this did not reach a level of statistical significance. However this finding may be biologically important. It has been

suggested that apoptosis may be involved in the decrease in cellularity associated with the resolution of granulation tissue to normal scar (4) and this was examined using *in situ* end labeling of fragmented DNA. Quantitation revealed a gradual increase in the percentage of apoptotic cells in hypertrophic scar tissue relative to their normal dermal controls as a function of time following the burn injury. This finding may be attributable to spontaneous resolution of hypertrophic scar to more normal or mature scar as is seen over time. However, the finding that there was an increase in the percentage of apoptotic cells in the hypertrophic scar tissue following the administration of interferon relative to normal dermis but that this was comparable prior to and following the termination of the administration of interferon, suggests that this effect may be partially induced by interferon.

Taken together these data strongly support the conclusion that IFN-α2b inhibits wound contraction and may be beneficial in the treatment of scar contracture in humans. This effect may be mediated through alterations in the production and functional interaction of fibroblast cytoskeletal proteins and/or through a decrease in proliferation or enhancement of programmed cell death of fibroblasts *in vivo*. It also emphasizes that the impact of the extracellular matrix proteins and soluble molecules should not be overlooked. Within the *in vitro* model system the effect of fibrogenic factors such as transforming growth factor-ß and platelet-derived growth factor are equally as important as the antifibrogenic factors (Chapter 3). The importance of "outside in" signaling from extracellular matrix proteins probably has been

underestimated but has recently become an area of intense investigation (3).

Confining the debate to whether wound contraction and scar contractures are generated by a myofibroblast (6) or treadmilling of fibroblast microfilaments (8, 9, 15) is an oversimplification of tissue remodeling. The recent elucidation of the role that extracellular agonists such as bradykinin, lysophosphatidic acid, epidermal growth factor, and platelet derived growth factor play in microfilament organization through Rho GTPase proteins (22, 24) suggests that these signals may be upstream to intracellular reorganization of cytoskeletal proteins. Integrin interactions with extracellular matrix proteins may have a bidirectional effect. They are required for fibroblasts to alter the organization of the extracellular matrix proteins such as collagen (10, 18). In addition the presence or absence of extracellular matrix components in turn alters the organization and expression of the cytoskeletal proteins, eg. the enhancement of α-SMA expression by heparin (4). Alterations of the extracellular matrix can therefore directly inhibit or enhance the ability of fibroblasts to contract a matrix. The correlation between the lack of decorin staining and presence of α-SMA within the guinea pig wound tissue suggests that the expression of α-SMA may be in response to the absence of decorin and possibly indicate a fibroblast whose ability to reorganize the collagen fibrils into fibres and fibre bundles has been frustrated. The lack of collagen fibres and fibre bundles (12), presence of α-SMA positive staining fibroblasts (5), and lack of decorin staining (19, 20) suggests that scar contractures may in fact result from the inability of

fibroblasts to organize the extracellular matrix into a more supple, pliable tissue. Scar contracture therefore may be a reflection of tissue rigidity and turgor associated with hyperhydration and increases in proteoglycans such as versican and biglycan (19, 20) which restricts the fibroblasts ability to align collagen fibrils in a manner that enhances the formation of fibres and fibre bundles. The renewed discussion suggesting that contraction in open wounds is based on polarized coordinated migration rather than granulation tissue (7) further undermines the theory that scar contractures are the result of myofibroblast contraction.

6.2 Future Research

The results and conclusions presented in this thesis provide support for the use of IFN-α2b in the treatment of scar contractures associated with HSc however many questions remain.

The lack of a model which reflects human scar contractures needs to be further pursued. With the availability of quantitative analysis of extracellular matrix components in HSc (20), the fibroblast-populated collagen lattice model system could be adapted to more closely approximate the microenvironment in scar tissue and assess whether these manipulations will directly effect contraction. Comparative analysis of HSc obtained from scar contractures relative to HSc in regions that do not demonstrate contraction in the same person, may shed light on whether the rigidity seen in both cases is what leads to the contractures or whether the fibroblasts themselves differ.

The expression of α-SMA in fibroblasts is unequivocally associated with pathologic tissue (Table 1, Chapter 2) yet the correlation with alterations in fibroblast function remains circumstantial. The technology exists to create a defined system where α-SMA expression can be manipulated while controlling all other variables to accurately infer the functional significance of α-SMA expression by fibroblasts. Transfection experiments to enhance or eliminate the production of α-SMA would allow for direct assessment of changes in cell behavior.

In both the *in vivo* animal model and the human tissue analysis there is evidence that the beneficial effect of IFN-α2b may at least

in part be mediated through the induction of apoptosis. The use of *in situ* end labeling of fragmented DNA allows for the localization and semi-quantitation of apoptotic cells within tissue however as with any experimental technique it also has limitation. To our knowledge this is the only report suggesting IFN-α2b induced apoptosis in fibroblasts and may be an important factor in maximizing the therapeutic efficiency in clinical application. This finding therefore demands more extensive and rigorous testing within more readily controlled *in vitro* conditions and the use of additional techniques to clarify the sequence of events leading to the induction of apoptosis.

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Patient Name	sex	age	TBSA	Biopsy Title	days/months post burn	VBSA	total # fibroblasts	SD
Furlotte, Cecile	M	49	30	H1	17 months	13	6.80	2.53
				H2	18 months	11	8.50	5.44
				H3	19 months	10	7.40	3.81
				H4	20 months	10	5.50	2.22
				H5	21 months	10	5.60	2.37
				H6	23 months	8	5.40	3.5
				H7	24 months	8	8.30	2.58
				H8	25 months	9	5.30	2.87
				N1	17 months	0	3.80	2.44
				N2	18 months	0	3.30	2.06
				N3	25 months	0	1.80	2.57
Goulet, Greg	M	26	70	H1	20 months	6	9.60	5.23
				H2	21 months	9	10.10	3.14
				H3	22 months	8	10.40	2.95
				H4	24 months	6	8.10	2.02
				H5	26 months	4	8.10	4.79
				H6	28 months	3	7.80	3.22
				N1	20 months	0	3.90	3.21
				N2	28 months	0	4.30	3.97
Melan, Landon	M	36	65	H1	4 months	10	19.80	6.65
				H2	6 months	10	11.20	5.51
				H3	8 months	5	9.40	4.35
				H4	10 months	3	8.00	4.16
				H5	12 months	2	9.10	2.33
				N1	4 months	0	3.60	2.67
				N2	12 months	0	6.40	2.55
Miller, Daryl	M	31	60	H1	21 months		5.40	2.01
				H2	22 months	8	4.60	1.65
				H3	25 months	8	6.00	3.86
				H4	26 months	7	5.20	3.05
				H5	28 months	7	4.20	1.75
				H6	30 months	6	8.70	7.12
				N1	21 months	0	6.40	6.20
				N2	30 months	0	5.70	3.95
Rawson, Joy	F	10	40	H1	11 months	11	10.90	4.33
				H2	15 months	5	11.70	4.37
				H3	17 months	5	7.90	1.52
				N1	11 months	0	7.70	3.80
				N2	17 months	0	8.40	4.72
Sabo, Ed	M	29	85	H1	8 months		8.80	2.62
				H2	11 months	7	8.10	5.38
				H3	12 months	7	5.90	2.42
				H4	14 months	6	5.20	1.69
				H5	16 months	5	4.30	1.83

Name	Sex			Code	Time			
				N1	8 months	0	3.00	1.56
				N2	16 months	0	3.20	1.81
Stock, Ernie	M	25	35	H1	6 months	9	8.60	3.47
				H2	8 months	9	7.50	2.84
				H3	11 months	8	11.10	5.61
				H4	12 months	8	9.10	4.82
				N1	6 months	0	6.40	4.77
				N2	12 months	0	4.30	2.63
Supervich, Richard	M	49	32	H1	4 months	12	8.60	5.19
				H2	6 months	12	9.80	11.60
				H3	8 months	10	9.40	5.60
				H4	11 months	11	8.10	3.67
				H5	12 months	9	3.20	3.05
				N1	4 months	0	1.90	2.92
				N2	12 months	0	5.10	4.95
Wang, Grace	F	46	20	H1	16 months	12	9.90	4.58
				H2	18 months	11	9.40	2.91
				H3	20 months	9	11.10	5.38
				H4	22 months	8	11.20	2.57
				H5	24 months	7	16.50	11.65
				N1	16 months	0	3.90	2.85
				N2	24 months	0	2.90	1.29
Akkinneah, Roger	M	42	3	G	6 days		19.40	0.30
				N	6 days	0	4.20	3.71
				HB	3 months	6	11.90	4.48
				HD	3 months	0	5.50	5.99
Amedjo, Samuel	M	41	40	G	17 days		9.30	5.66
				N	17 days	0	6.90	5.72
				HB	3 months	9	15.30	7.65
				HD	3 months	0	3.20	2.53
Dillon, Micheal	M	35	18	G	7 days		3.20	1.40
				N	7 days	0	2.30	1.42
				HB	12 months	1	6.40	3.31
				HD	12 months	0	3.50	3.50
Hanke, Ralph	M	28	85	G	75 days		19.80	6.66
				N	75 days	0	12.60	7.01
				HB	12 months	12	10.00	3.30
				HD	12 months	0	8.20	4.78
Lowman, David	M	49	25	G	4 days		11.40	8.86
				N	4 days	0	4.60	3.31
				HB	4 months	6	9.80	0.92
				HD	4 months	0	8.70	9.17
Mundt, Albert	M	67	5	G	5 days		9.10	5.74
				N	5 days	0	2.80	1.48
				HB	2 months	0	8.60	10.91
				HD	2 months	0	10.60	6.69

mean % + ASMA	SD	mean % + TUNEL	% + TUNEL relative to normal	
17.02	14.34	38.71	269.94	Abbreviations
23.66	19.72	45.57	317.78	H - hypertrophic scar
12.44	13.93	34.31	239.26	N - normal dermis
29.80	22.94			G - granulation tissue
26.48	23.20	31.96	222.87	HB - healed burn
12.42	15.28	23.30	162.48	HD - healed donor
20.52	13.68	18.60	129.71	SD - standard deviation
25.58	19.46			
16.00	19.36	19.51	100.00	n =10 for column H, J, and L
9.00	16.63	14.43	100.00	
8.10	21.07	9.09	100.00	all counts obatined from high power field (100 x lens with 10 x ocular)
47.55	23.48	33.70	192.13	
45.83	30.64	31.86	181.64	
15.76	24.62	75.16	428.51	mean % + ASMA obtained by
7.44	10.49	44.54	253.93	calculating % + fibroblasts
24.72	17.07	30.15	171.89	per field as counts were
13.90	15.66	15.45	88.08	obtained on the same
0.00	0.00	17.54	100.00	section, therefore the mean
3.08	9.73			and SD were calculated after
39.67	34.98	45.60	166.00	mean % + TUNEL was
21.89	15.77	59.00	214.60	calculated by obtaining the
6.56	11.90	29.00	105.60	mean # of cells that were
14.81	17.73	29.50	107.50	positive and the mean total
8.93	19.30	32.50	118.40	# of cells from the serial
10.17	16.56	33.90	100.00	section and then calculating
5.92	8.06	21.00	100.00	the % +
4.17	9.00	71.76	599.00	
3.43	7.35	32.47	271.04	
0.00	0.00	32.26	269.28	
5.00	10.00	48.00	400.67	
17.00	19.27	26.19	218.61	
21.20	15.06	14.43	120.47	
3.18	6.97	17.14	100.00	
9.42	18.45	6.82	100.00	
16.66	25.33	22.70	84.20	
6.12	6.26	29.92	110.98	
7.68	12.17	20.54	75.74	
2.00	6.32	33.63	100.00	
0.00	0.00	20.29	100.00	
65.22	28.88	41.38	66.29	
27.49	35.01	43.09	69.03	
13.53	11.26	47.30	75.78	
33.14	24.20	34.57	55.38	
7.08	9.63	53.13	85.12	

11.67	15.31	72.22	100.00
14.86	31.64	52.63	100.00
12.61	18.77	25.00	57.33
10.69	15.04	35.66	81.77
26.40	32.71	37.38	85.71
26.90	17.80	25.58	58.66
14.89	20.09	66.67	100.00
6.43	11.45	20.55	100.00
20.71	13.63	20.51	97.20
11.25	13.86	25.43	120.52
17.71	16.55	20.58	97.54
14.73	9.12	14.49	68.67
24.73	23.64	12.50	59.24
6.00	15.78	13.63	100.00
25.71	24.26	28.57	100.00
14.56	20.65	1.54	4.79
5.86	9.45	23.71	73.77
14.15	16.03	7.56	23.52
14.50	17.70	27.12	84.38
7.96	8.64	43.95	136.75
0.00	0.00	26.79	100.00
0.00	0.00	37.50	100.00
6.85	6.90	13.14	62.13
4.76	11.00	21.15	100.00
0.67	2.11	8.85	41.84
0.91	2.87		
3.92	8.30	48.94	87.50
1.11	3.51	55.93	100.00
12.23	13.25	13.68	24.46
2.00	6.32	20.83	37.24
15.83	32.02	29.73	148.65
2.00	6.32	20.00	100.00
12.62	12.09	22.22	110.10
20.12	26.81	4.92	24.60
27.89	19.44	15.95	126.89
5.35	7.79	12.57	100.00
37.30	27.33	33.98	270.33
5.54	7.41	7.02	55.85
0.77	2.43	28.10	92.13
2.26	4.97	30.50	100.00
9.61	11.47	14.10	46.23
4.40	10.71		
0.00	0.00	10.43	24.77
33.30	10.54	42.10	100.00
1.28	4.05	39.06	92.78
1.91	4.03	29.31	69.62