TGF-β1 REGULATION OF α-SMooth MUSCLE ACTIN EXPRESSION IN FIBROBLASTS IS DEPENDENT ON THE DEFORMABILITY OF THE SUBSTRATE

by

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A Thesis submitted in conformity with the requirements for the Degree of Master of Science Graduate Department of Dentistry

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ABSTRACT

Wound contraction is a fundamental process in wound repair and is required for closure. but the interactions between the inflammatory cytokines that regulate this process and the cytoskeletal elements in fibroblasts that provide the contractile forces are poorly understood. I examined the effect of TGF-β1 on the ability of human gingival fibroblasts to contract collagen gels in vitro and on the expression of the putative fibroblast contractile marker, α-smooth muscle actin (α-SMA). TGF-β1 (10 ng/ml; 3 days) increased α-SMA protein and mRNA 2-3 fold, as determined by western and northern blots and normalised for β-actin content. Western blots of cells in anchored collagen gels also showed a TGF-β1-induced increase of α-SMA content but the effect was reduced compared to collagen-coated plastic. In floating collagen gels, TGF-β1 exerted no significant effect on α-SMA protein. Notably, TGF-β1 increased the rate of contraction in anchored and floating collagen gels in serum-free cultures by 50% and 30% respectively but the difference was not evident in serum-containing cultures. Using an ELISA, active and latent forms of endogenous TGF-β1 were detected in all 3 models. Addition of exogenous TGF-β1 stimulated its own production. The highest levels of endogenous TGF-β1 were detected in monolayer cultures and the lowest levels in floating gels. Blocking α2 and β1 subunits with monoclonal antibodies abolished the effect of TGF-β1 on α-SMA expression. Collectively these data indicate that TGF-β1 may regulate wound contraction by affecting the cellular content of the contractile protein, α-SMA and that generation of intracellular tension may regulate expression of specific cytoskeletal genes involved in cell contraction.
I would like to thank and acknowledge my supervisor Dr. Chris McCulloch for his invaluable support, encouragement, guidance and words of advice that were always given with a smile. I would like to take this opportunity to express my endless gratitude to Chris for all that he has done for me during my graduate education. I consider it a privilege and an honor to know him and to have worked with him. I also wish to acknowledge Dr. Jack Ferrier and Dr. Sela Cheifetz as members of my advisory committee for their helpful comments and constructive criticisms.

I must also extend my thanks to all the members of MRC Group with whom I had the pleasure of working. I am specifically grateful to Pam Arora for her extensive assistance and constructive advice throughout this project; Laura Luo for northern blots; and Harry Moe for helping me with anything whenever I needed it and for always being there for me.

My deepest appreciation goes to my understanding and loving family. I am indebted to my mother, brother and grandparents for their constant encouragement, support and most importantly, their faith in me. Finally, I dedicate this thesis to my husband, Reza, for his love, patience and support throughout the two years of this program and for his willingness to go through it again.

I also acknowledge financial support received from the MRC Group in Periodontal Physiology Group Grant and University of Toronto Open Master’s Fellowship.
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II: REVIEW OF THE LITERATURE

1. WOUND HEALING

1.1. Overview

Wound healing occurs as a consequence of well-organized, interrelated events and involves a variety of cell types acting in concert to re-establish the integrity of injured tissues. The overall healing response can be divided into three overlapping phases including: 1. inflammation; 2. granulation tissue formation and contraction; 3. matrix maturation and remodeling. To provide an overview of my research project, I will briefly provide a historical review of these three phases before describing in detail the focus of my research.

1.1.1. Inflammation

Hemostasis Tissue injury results in damage to blood vessels which leads to exposure of subendothelial structures. Connective tissue components of the subendothelium (e.g. collagens type IV and V, laminin and proteoglycans) are known to promote platelet aggregation and activation (Barnes et al. 1980, Chiang et al. 1980, Weksler et al. 1988). Activated platelets release a variety of products such as, von Willebrand factor, platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β) and thromboxane A₂ (Weksler et al. 1988) and are also involved in activation of the coagulation cascade leading to thrombin formation. Thrombin catalyzes the formation of fibrin from fibrinogen (Wahl et al., 1992). Fibrin strands form a mesh between aggregated platelets and trapped red blood cells thereby forming the clot that seals off the injury and prevents further bleeding.
polymorphonuclear leukocytes is an early response after tissue injury. Factors released during platelet aggregation and clotting include a group of vasodilators (e.g. serotonin, bradykinin, histamine, arachidonic acid metabolites) and inflammatory mediators (e.g. interleukin-1 [IL-1], tumor necrosis factor-alpha [TNF-α]) which influence capillary permeability and promote leukocyte adhesion to endothelial cells (Osborn et al. 1990, Pohlman et al. 1986). Further, the local release of a variety of leukocytic chemoattractants during hemostasis (e.g. kallikrein, plasminogen activator, PDGF) serves as a migration signal for increased cell adherence (Malech, 1988).

Monocyte Chemotaxis Following an initial trauma and after neutrophil accumulation, monocytes/macrophages accumulate in the inflammatory site, presumably in response to chemoattractants (Ohura et al., 1987). These cells participate in debridement, microbicidal events and orchestration of several later events involved in tissue repair (Wahl et al., 1992). For example one of the most potent chemotactic agents that can be found in an inflammatory site is transforming growth factor-beta (TGF-β), an important cytokine that is released by monocytes and also by platelets, neutrophils and lymphocytes (Grotendorst et al., 1989). TGF-β stimulates monocytes to produce a variety of cytokines including IL-1 which contribute to the activation of inflammatory network (Wahl et al., 1987).

Macrophages and monocytes are also important in matrix remodeling. Damaged tissue at the site of injury must be degraded before healing can proceed, a requirement that necessitates collagen-degrading enzymes. Collagen is one of the major connective tissue components in damaged tissue and the enzyme collagenase plays a pivotal role in collagen degradation. Macrophages and monocytes are a source of collagenase and along with neutrophils and fibroblasts, they contribute to the degradation and removal of collagen from damaged tissue (Wahl et al., 1992).
The formation of granulation tissue and the provisional matrix, especially the cross-linking and reorganization of nascent collagen is a critical step in wound healing. One of the key cells in collagen synthesis and remodeling is the fibroblast. Inflammatory products released during hemostasis by activated platelets, endothelial cells, infiltrating monocytes and lymphocytes (e.g. PDGF, fibroblast growth factor [FGF], TGF-β) encourage fibroblast migration into the wound area and promote fibroblast proliferation (Wong and Wahl, 1989). Migrating fibroblasts play a central role in depositing the fibronectin-rich, provisional matrix (Grinnell et al., 1981; Kurkiren et al., 1980). While we currently do not have a detailed understanding of the regulatory mechanisms involved in the production of granulation tissue by fibroblasts, in vitro studies have suggested a role for thrombin (Mosher and Vaheri, 1978), epidermal growth factor (EGF) (Chen, 1977) and TGF-β (Ignotz and Massague, 1986; Roberts et al., 1986) in regulating the formation of collagen, fibronectin and other provisional matrix components by fibroblasts.

The deposition of a provisional matrix is followed by wound contraction, another vital aspect of wound healing and the principal focus of this thesis. Wound contraction is defined as the mechanism by which the edges of the wound are drawn toward the center. This centripetal movement is likely due to forces generated in the wound and reduces the size of the wound so that smaller amounts of connective tissue deposition and epithelialization are required to reconstitute the lost tissue (Clark, 1988; Mast, 1992). As fibroblasts migrate into the wound space and deposit the provisional matrix, they undergo an alteration of cell phenotype by acquiring morphological features of smooth muscle cells (Gabbiani et al., 1971). These cells form several types of cell to cell and cell to matrix linkages (Ryan et al., 1974; Gabbiani et al., 1978; Singer et al., 1984) and they develop the ability to exert tractional force on the matrix which in turn leads to wound contraction. The
Demonstrations about the smooth muscle have been demonstrated in vitro by exposing isolated strips of granulation tissue to vasoconstrictors such as epinephrine and angiotensin (Gabbiani et al., 1972).

1.1.3. Maturation of Provisional Matrix and Remodeling

The last and longest step of healing involves the replacement of the provisional matrix (rich in fibronectin, proteoglycans and types I, III and IV collagen) by a more mature fibrous matrix. This process is characterized by the continued synthesis, cross-linking and remodeling of collagen, which gives rise to large bundles of collagen type I, thereby providing the tissue with increasing tensile strength. Notably, growth factors such as TGF-β can modulate fibroblast remodeling of connective tissue (Ignottz and Massague, 1986; Roberts et al., 1986). Since the interactions between cells and their substrate in the granulation tissue are likely to be important in wound closure, I will focus on the formation of granulation tissue in the next section of this review.

1.2. Granulation Tissue

As described above, granulation tissue consists of a dense, mixed population of inflammatory cells, fibroblasts and newly formed blood vessels embedded in a provisional matrix mainly composed of collagen and fibronectin. The formation of granulation tissue involves several correlated events that I will outline below.

1.2.1. Re-epithelialization

When the integrity of the epithelium is compromised by injury, it is critical for survival of the organism that access to the underlying connective tissue by foreign environmental factors (e.g. bacteria) is blocked immediately. The blood clot that forms shortly after injury acts as a temporary barrier which is later covered by migrating and
Migration and proliferation of the epithelial cells are independent of each other as migration starts within a few hours after injury; proliferation begins within 1-2 days (Krawczyk, 1971, Winter, 1972). The stimuli for cell migration and proliferation are unknown, however several mechanisms have been suggested including the elaboration of chemotactic factors, contact guidance or the loss of neighbouring cells (Stenn et al., 1988). Among the growth factors that have been studied in the context of wound healing, EGF is a leading candidate since it has potent chemoattractant and mitogenic effects on several different cell types and EGF receptors are abundant on epithelial cells (Nanney et al., 1984). In addition, its central role in wound healing has been demonstrated in several studies (e.g. Laato et al., 1986, Brown et al., 1986).

1.2.2. Fibroblast Migration and Proliferation

Successful wound repair depends to a large extent on the proliferation of local fibroblasts and the regulated migration of fibroblasts into the wound space. Fibroblasts are not only important for connective tissue deposition but migrating fibroblasts may also be required for matrix contraction and remodeling. Migrating cells can exert tractional forces on the surrounding substrate and can assist in the alignment of collagen bundles (Harris et al., 1981; Ehrlich and Rajaratman, 1990; Rudolph et al., 1992). The signals for migration and proliferation of fibroblasts are not well delineated but growth and chemotactic factors released initially by platelets and later by monocytes/macrophages at the site of the injury are likely to be important. Growth factors released from the granules of activated platelets such as PDGF, EGF, FGF, TGF-α and TGF-β are among leading candidates for regulation of fibroblast migration and proliferation (Sporn and Roberts, 1986). PDGF has both mitogenic and chemotactic effects on fibroblasts: indeed, it has been described as the most potent mitogen in serum for cells of mesenchymal origin including fibroblasts and smooth
of PDGF has been demonstrated for neutrophils, monocytes (Deuel, 1982) and fibroblasts (Deuel et al., 1984). FGF has also been proposed as an important wound healing cytokine based on its mitogenic effect on fibroblasts and endothelial cells (Gaspodarwicz, 1979; Duthu et al., 1980).

1.2.3. Connective Tissue Deposition

The deposition of granulation tissue is an important function of fibroblasts in response to different stimuli. Granulation tissue provides a substrate on which endothelial and inflammatory cells and fibroblasts themselves can migrate into the wound. The initial or provisional matrix contains large amounts of fibronectin (Grinnell, 1981) and may serve as a template for collagen deposition (McDonald et al., 1982). Fibronectin in granulation tissue is often associated with fine fibrils of type I and type III collagen (Repesh et al., 1982). During matrix maturation fibronectin is gradually replaced by collagen type III. Later on collagen type I becomes the major component of the mature matrix (Clark, 1988). As the remodeling of collagen proceeds, collagen type I becomes more heavily cross-linked and fibrils grow in size to form fibrous bundles, thereby increasing the tensile strength of the wound.

1.2.4. Angiogenesis

The formation of new blood vessels is necessary for the survival and elaboration of granulation tissue. The mechanisms involved in activation of angiogenesis during tissue repair are poorly understood, however loss of contact between endothelial cells and their basement membrane is thought to be an important stimulus (Mardi and Pratt, 1988). Upon activation, collagenase released by neutrophils and monocyte/macrophages degrades basement membrane and the endothelial cells are then capable of initiating neovascularization (Mainardi et al., 1980). Activated endothelial cells can also produce
endothelial cells start to migrate into the wound space, probably in response to several chemotactic and mitogenic factors released by inflammatory cells and at these sites form new blood vessels. A number of factors have been implicated as regulators of endothelial cell migration and/or proliferation including TNF-α (Folkman and Klagsburn, 1987), TGF-α (Schreiber et al., 1980), and TGF-β (Madri et al., 1988). Newly formed blood vessels undergo further remodeling and maturation in response to several stimuli including hypoxia and shearing force generated by circulation. Shearing force is capable of affecting endothelial cells in many ways including modification of cell to cell and cell to matrix interactions, and by up-regulation of growth factors such as PDGF (Risau, 1997). As described briefly above, the integrated process of wound closure consists of several steps. Each step plays an important role in the final outcome of the process which is restoration of tissue architecture. Since the emphasis of this literature review is on cell-mediated wound contraction and the regulation of these processes, in the next section I will focus on cell contraction and the requirements for wound closure.

1.3. Requirement for Cell Contraction in Wound Closure

1.3.1. Wound Closure

A normal healing response includes epithelialization, connective tissue deposition and contraction. The contribution of each of these three steps to wound healing depends on the type of wound which in turn is determined by the type of therapeutic intervention. Wounds can be classified into two groups:

i. Closed wounds- closure is obtained shortly after injury and healing is by primary intention. Connective tissue deposition is the main process involved in this type of healing.
case the main mechanism of healing is by contraction. Thus open wounds are characterized by their ability to contract and to decrease the area of the wound. The open wound is repaired by connective tissue deposition and epithelialization.

### 1.3.2. Cell Mediated Force

The origin of the force required for contraction has been debated for a long time. Here I will review the different theories that have been proposed and I will focus on two main theories of wound contraction. Both of the theories point to granulation tissue fibroblasts as the central force-generating elements, but one of them highlights the contribution of specialized fibroblasts, while the other refers to the more general issue of fibroblast locomotion.

**Theories of contraction** Wound contraction was formerly believed to be generated by the developing collagen fibers in the granulation tissue (Chu, 1955). This idea was later questioned by Abercrombie et al. (1956) who demonstrated wound contraction in scorbutic pigs and instead suggested the “pull theory”. In this model, cells throughout the granulation tissue generate the force. In 1956, Edwards introduced the “push theory” which suggests that the force was generated in the skin surrounding the defect. Evidence to negate this theory came from experiments in which excision of the wound edges during wound healing did not affect wound contraction (Cuthbertson, 1959). Grillo et al. (1958) introduced the “picture frame theory” which suggested that the force is generated by the cells at the edge of the wound. Notably, these experiments used animals with a mobile skin (guinea pigs) which may behave quite differently than the healing of human skin. Recently, Gross and co-workers (1995) provided further evidence for the picture frame theory in which granulation tissue formation was prevented in a domestic pig model. The domestic pig is often used as a
i. Contraction of actin-rich fibroblasts ("myofibroblasts") In 1971, Gabbiani and co-workers reported the presence of modified fibroblasts in granulation tissue. These phenotypically modified fibroblasts showed ultrastructural features similar to both fibroblasts and smooth muscle cells and were therefore called myofibroblasts. Since myofibroblasts were present in large numbers during the time period when contraction was most vigorous, these specialized cells were suggested as generators of the force required for contraction. Further in vitro studies revealed that in response to vasoconstrictors, granulation tissue contracts like smooth-muscle (Gabbiani et al., 1972; Ryan et al., 1974). Using electron microscopy, myofibroblasts were characterized by well-developed actin microfilaments (Gabbiani, 1978) and several types of cell to cell and cell to matrix connections (see below). These connections are likely involved in synchronizing the tensional forces from different regions throughout the whole cell and in transmitting intracellular forces to the surrounding matrix (Ryan et al., 1974; Gabbiani et al., 1978; Singer et al., 1984). Myofibroblasts may also exhibit multiple indentations in the nucleus similar to those in muscle cells (Franke et al., 1969). There is convincing evidence to support the view that myofibroblasts are involved in active phase of wound contraction (Gabbiani et al., 1971; Rudolph et al., 1979; Bertolami et al., 1979; McGrath and Hundahl, 1982). In contrast, using "tight skin mice" (a specific genetic strain that exhibits a delay in wound closure), Hembry (1986) was not able to show a correlation between the relative abundance of myofibroblasts and the period of maximal wound contraction. However, caution must be used in interpretation of the information obtained from this genetically altered animal and its relevance to normal human wound contraction.
discussed at length. Some investigators believe that as all fibroblasts are able to generate force and reorganize their substrate (Harris et al., 1981; Ehrlich et al., 1988), then it follows that all fibroblasts are essentially of the same type (i.e. no sub-types). On the other hand, electron microscopic studies have shown that fibroblasts acquire distinct ultrastructural and functional features under different conditions. Also the identification of defined cytoskeletal differentiation markers in myofibroblasts which will be discussed later, further supports the idea of fibroblast heterogeneity (Sappino et al., 1990; Schmitt-Graff et al., 1994).

ii. Fibroblast locomotion Studies conducted by Ehrlich (1988) have suggested that the cell-generated contractile forces necessary for wound closure do not require a specialized fibroblast; instead, cell locomotion can provide the force for contraction. In an earlier study performed by Ehrlich and co-workers (1977), myofibroblasts could not be detected in full-excision wounds in rats any earlier than 7 days, at which time the wounds had already contracted by as much as 50%. On the other hand, migration of fibroblasts into the healing wound starts as early as 2 to 3 days (Billingham et al., 1956). Harris and co-workers (1981) demonstrated that non-specialized migrating fibroblasts can exert tractional force on their substrate and act as single units to reorganize the connective tissue fibers. In dynamic models of cell-generated contractile forces, motile fibroblasts are the prominent cell type during the period of contraction, whereas myofibroblasts may become prominent at the end of the contractile process when the collagen fibers can be reorganized no further and cell to cell contacts develop (Ehrlich and Rajaratman, 1990). Further, Eastwood et al., (1996) have shown that most of the force generated by fibroblasts seeded into collagen gels was produced during the stage of cell attachment and migration (first 24 hours) when there was no evidence for the presence of myofibroblasts. Thus the need for specialized “myofibroblasts” in wound contraction is uncertain. The major difference between
linked to the idea that the myofibroblast is a specialized fibroblastic phenotype. In essence the myofibroblast is considered as the central force-generating element which probably acts in multicellular units and the mechanism is based upon coordinated cell contraction (Ryan et al., 1974). In contrast, the fibroblast locomotion theory suggests that the movement of individual fibroblasts in the matrix causes the rearrangement of the surrounding matrix. Since fibroblasts are attached to their substrate via several types of specialized adhesion molecules (e.g. integrins, see below), they can rearrange the matrix as part of cell migration. As much of the data used to assess each theory has come from in vitro studies of fibroblast-populated collagen gels, the relevance to the in vivo situation must be established carefully before interpreting these findings. Further, the appearance of specialized fibroblasts (myofibroblasts) during wound healing as well as fibrocontractive disorders strongly suggests their pivotal role in tissue contraction and remodeling in vivo.

Grinnell (1994) put forward a new explanation by combining these two previously described theories. He suggested that migration of fibroblasts into the wound space provides the tractional forces for differentiation of myofibroblasts; once the mechanical stress is relieved, cells will switch to a non-proliferating phenotype and begin to regress, even in the presence of growth factors (Nakagawa et al., 1989a and 1989b; Nishiyama et al., 1989, Grinnell and Nakagawa, 1991).

To study wound contraction and other related phenomena, an in vitro model was developed in which the contraction of fibroblast populated collagen lattices can be studied (Bell et al., 1979). This model has been used to compare the generation of contractile forces by myofibroblast contraction or fibroblast locomotion. Fibroblasts cultured in collagen matrices acquire phenotypic characteristics similar to fibroblasts in vivo which is not typically observed for cells in monolayer cultures (Elsdale and Bard, 1972; Bell, 1979;
include the development of cellular reticular arrangement within the gel and the extension of this arrangement into a 3-dimensional, tissue-like network. When considering the interpretation of data from these models, it should be noted that wound repair in vivo includes a complex series of events involving the interaction of inflammatory cells, fibroblasts, endothelial and epithelial cells, growth factors, cytokines and extracellular matrix (ECM) components. In contrast, the simulated model of wound contraction in vitro is a greatly simplified environment that uses a single cell type and collagen as its substrate. However in my opinion the morphological similarities of fibroblasts in collagen matrices to cells in vivo and their arrangement into a 3-dimensional, tissue-like network appears to substantiate the use of collagen gels as a suitable model for studies of wound contraction.

1.3.3. General Mechanism of Cell Contraction

The cytoplasm of fibroblasts contains cytoskeletal contractile structures rich in actin and myosin which are thought to mediate cell contraction. Here I will introduce the mechanism of cellular force generation in non-muscle cells including the interactions between the contractile proteins actin and myosin.

The presence of actin and myosin in most types of eukaryotic cells and the similarity of their structure to actin and myosin in muscle cells suggests that the mechanism of generating force in muscle cells is universal (Clarke and Spudich, 1977). In non-muscle cells, filaments of actin and myosin form loosely organized networks that reveal the same basic structural features identified in muscles including the interaction of actin filaments with the heads of myosin fibers (Niederman and Pollard, 1975). This suggests that, at least in theory, the contractile proteins of non-muscle cells can generate tension and motion by the same sliding filament mechanism used in muscle (Pollard, 1981).
ATP and $\text{Mg}^{2+}$ can mediate muscle contraction (Pollard and Weihsing, 1974). Muscle and non-muscle myosins are able to form bipolar filaments under physiological conditions in vitro (Pollard et al., 1978). Actomyosin regulatory proteins such as tropomyosin, myosin light chain kinase and calmodulin have been identified in non-muscle cells (Darnell et al., 1986). Further, it has been demonstrated that the role of myosin light chain kinase in fibroblast contractility is similar to its role in smooth muscle contraction (Kolodney and Elson, 1993).

2. MOTORS FOR CELL CONTRACTION

2.1. Actin Cytoskeleton

The cytoskeleton is a dynamic network composed of three main types of filaments including microfilaments, microtubules and intermediate filaments. The interconnected function of these components is essential for many diverse cellular activities and provides the cell with the ability to change shape and to achieve directed movements. In this section, because of the pivotal role of actin in cell motility and contraction, I will focus on actin microfilaments, their functions and regulatory mechanisms.

2.1.1. Function

Actin, along with a number of actin-binding proteins is involved in a wide variety of motility-dependent processes including cell locomotion, cytoplasmic streaming and transport, secretion, phagocytosis, cytokinesis and cell shape changes (Weeds, 1982, Cramer et al., 1994). Amoeboid movements of cells have been described as continuous cycles of actin assembly, cross-linking (gelation), a decrease in gel structure (solution), contraction and partial disassembly of actin filaments (Taylor and Condeelis, 1979). Stossel
which there is regulated assembly and disassembly of actin filaments. He suggested that a focal defect occurs in the cortical actin gel in response to migratory stimuli. The hydrostatic pressure causes the "sol" part of the cell to push out through the defect and this produces the force necessary for movement. Microfilaments in non-muscle cells are mainly composed of actin bundles in association with other contractile proteins such as myosin and are potentially contractile (Isenberg et al., 1976). A functional cytoskeleton is also necessary for phagocytosis, a process essential for the remodeling of extracellular matrix by fibroblasts.

Actin polymerization is regulated by actin binding proteins, ATP and divalent cations (Pollard and Cooper, 1986). Cycles of actin assembly and disassembly provide cells with the ability to extend lamellipodia rich in microfilaments that can further extend, surround and internalize extracellular materials (Garant, 1976). Actin microfilaments are also involved in transport of receptors over the cell surface, cytoplasmic flow, vesicle transport and propulsion of intracellular pathogens (Cramer and Mitchison, 1994). The various shapes that cells exhibit, including cell rounding, cytokinesis, and cell spreading, as well as surface extensions such as the sheet-like process at the leading edge of a crawling cell are the result of dynamic turnover of actin filaments (Alberts et al., 1992). Contractile bundles of actin and myosin are involved in cell attachment, since they are associated with the plasma membrane at sites of focal contacts where the cell is closely attached to the extracellular matrix (Byers and Fujiwara, 1982, Langanger et al., 1986).

2.1.2. Actin Structure

The mammalian actin family is composed of six isoforms including β- and γ-non-muscle actins which are expressed ubiquitously by all cells and are known as cytoplasmic actins. In contrast, γ-smooth muscle, α-cardiac, α-skeletal, and α-smooth muscle actin are considered tissue-specific (Garrels and Gibson, 1976; Vanderkerckhove and Weber, 1978).
The genes encoding various isoforms have distinct transcriptional factors (Paradis et al., 1996; Hautmann et al., 1997). The different actin isoforms are identical in about 95% of their amino acid residues but as a group, cytoplasmic non-muscle actins are more similar to each other than they are to muscle actins. The most variable region of the actin molecule is the N-terminal 10 amino acid residues (Vanderkerckhove and Weber, 1978). This region is known to play an important role in regulation of actin polymerization in-vivo and in-vitro (Chaponnier et al., 1995). Non-muscle actins as well as muscle actins polymerize to form helical filaments which are identical in structure as judged by electron microscopy (Hartwig and Stossel, 1975). Actin filaments are all capable of binding myosin reversibly and in the presence of ATP and myosin exhibit Mg-ATPase activity (Gordon et al., 1976). Despite the structural similarities between different actin isoforms, a growing body of evidence suggests the presence of functional diversity in this family. Expression of different isoforms varies during cell development and is highly regulated both spatially and temporally (Rubenstein, 1990; McHugh et al., 1991). It has also been suggested that some pathologic and physiologic conditions are associated with the expression of a muscle isoform of actin, α-smooth muscle actin in fibroblasts. In some tissues such as periodontium, α-smooth muscle actin is expressed constitutively (Arora and McCulloch, 1994). This actin isoform may mediate cell contraction (Sappino et al., 1990).

The molecular structure of actin filaments has been established using X-ray diffraction and electron microscopy. According to a model based on the 3-dimensional reconstruction of a single actin filament, the filament is built as a double-stranded helix in which a single molecule of ATP is bound to each actin subunit (Mornet and Ue, 1984). In non-muscle cells it has been shown that a large proportion of actin is in the form of monomers that can assemble to form filaments and adopt a large variety of 3-dimensional
steps, including activation, nucleation, elongation and annealing which are precisely regulated by a number of proteins (actin binding proteins, ABP), nucleotides and divalent cations (Pollard and Cooper, 1986). ABPs are classified according to their function (Schliwa, 1981; Weeds, 1982; Craig and Pollard, 1982; Stossel, 1984) and can be classified into two groups: one group of proteins promotes the formation of actin bundles or isotropic gels, whereas the other group acts in the opposite direction to disrupt the actin bundles, or retard the incorporation of actin monomers into filaments.

Adenine nucleotides are also important in regulation of actin polymerization. Actin monomers can bind to both ATP and ADP, and actin polymerization is associated with the hydrolysis of actin-bound ATP to ADP (Stossel, 1985). The type of nucleotide bound to actin monomers may determine the rate and extent of polymerization (Pollard, 1984). Ca\(^{2+}\) can stimulate muscle contraction by a negative control mechanism that acts through a tropomyosin-troponin complex (Mannherz, 1976). However this method of regulation has not been shown yet in non-muscle cells. Both in muscle and non-muscle cells, Ca\(^{2+}\) and myosin light-chain kinase activity are regulated through another protein, calmodulin (Yerna et al., 1979). Although tropomyosin has been found in non-muscle cells, its action has not yet been firmly established. It may regulate the severing activity of actin binding proteins such as gelsolin by inhibiting actin turnover in stress fibers (Ishikawa et al., 1989). Notably, actin filaments can bind together to form large bundles of actin and other actin-related proteins (tropomyosin, \(\alpha\)-actinin) into large sarcomeric structures known as stress fibers which are thought to be the contractile elements in the cell (Lazarides, 1975a and 1975b; Gordon, 1978; Goldman et al., 1979, Sanger et al., 1980).
The assembly of actin filaments into stress fibers, 0.1-0.2 μm in diameter, is enhanced in well-spread cells in vitro, particularly fibroblasts. Stress fibers are predominant near to the ventral plasma membrane of well-spread fibroblasts (Clark and Spudich 1977). Stress fibers are remodeled during the cell cycle, disappear at mitosis and may play a structural role as well as roles in cell adhesion and motility. Attachment of the cells to the substrate triggers their reorganization (Goldman et al., 1976) and they dissociate both in mitotic cells and in transformed cells. Actin filaments in stress fibers are associated with myosin (Byers, 1982) and accessory proteins such as tropomyosin (Lazarides, 1975a), α-actinin (Lazarides, 1975b), filamin (Wang et al., 1975), myosin light chain kinase (De Lanerolle et al., 1978) and vinculin (Geiger, 1979). The stress fiber proteins are arranged in an alternating periodic order similar to that seen in muscle sarcomeres (Langanger et al., 1986) and are proposed to be potentially contractile (Isenberg et al., 1976). Rapidly motile cells such as macrophages and fibroblasts growing out of explants show a diffuse distribution of actin and myosin. However, using poorly understood mechanisms, cell adhesion to the substrate and spreading convert the diffusely organized actin filaments into the much more tightly packed and well organized stress fibers (Herman et al., 1981; Darnell et al., 1986).

Stress fibers are highly concentrated in the peripheral cytoplasm (cortex) of many cells and are associated with specialized sites at the plasma membrane termed focal adhesion plaques (Alberts et al., 1992). It is thought that the interaction of myosin with actin in stress fibers can generate force and exert tension on the plasma membrane and consequently on the ECM (Langanger et al., 1986). Certain situations (in vitro or in vivo) are known to promote the reorganization of stress fibers. Fibroblasts grown on rigid substrates in vitro develop stress fibers that are composed mainly of actin as shown by
et al 1981). Further, fibroblasts cultured in 3-dimensional collagen matrices that are attached to the underlying surface acquire a bipolar morphology in which the arrangement of stress fibers is parallel to the long axes of the cells (Bellows et al. 1981, Nakagawa et al., 1989a and 1989b). On the other hand, no stress fibers are seen in cells cultured on weakly adhesive substrates (Willingham et al., 1977). Stress fibers in vivo have been described in aortic endothelial cells of animals with hypertension (Gabbiani et al., 1975), in fibroblasts from wound healing (Gabbiani et al., 1971) and pathologic situations characterized by fibrosis (Sappino et al., 1990), suggesting the role of mechanical stress in their formation. Burridge (1981) proposed that the formation of stress fibers is a response to generation of tension. This response may be an attempt by the cell to pull against a point of strong adhesion. As suggested above, the arrangement of actin and myosin as well as actin-associated proteins in stress fibers resembles the structure of muscle myofibrils. The contractility of stress fibers has been demonstrated in detergent-treated cell models after addition of Mg-ATP and is consistent with the sliding filament mechanism found in muscle cells (Kreis and Birchmeier, 1980).

2.1.4. Mechanisms of Fibroblast Contraction

The presence of myofibril-like, contractile structures composed of actin and myosin bundles in fibroblasts under tension suggests their ability to undergo isotonic or smooth muscle-like contractions. However, this has not been demonstrated in vivo probably because the tight adhesions to ECM and the completely rigid ECM conditions that prevent shortening in vitro are dissimilar from the in vivo situation. Instead, conditions favoring isometric “contraction” or the generation of intracellular tension may be more prominent (Burridge, 1981). Isotonic contraction by fibroblasts have been demonstrated in vitro by stress-relaxation of anchored gels. With the release of an anchored gel, fibroblasts
stress fibers which in turn mediates the smooth muscle-like contraction of collagen matrices (Mochitate et al., 1991; Tomasek et al., 1992). If intracellular tension at different levels is to be studied, there is a need for cell culture models in addition to monolayer cultures and floating collagen gels. As the contractile potential of stress fibers requires transmission of intracellular forces to the extracellular matrix, in the next section I will focus on cell-matrix interactions and their role in force transmission.

2.2. Cell-Matrix Interactions

Cell-ECM attachments are thought to be important in both generating intracellular tension and in transmitting tensile forces to the extracellular matrix. The generation of intracellular tension thus plays a critical role in tissue remodeling during wound healing.

2.2.1. Wound Contraction

This process is a result of dynamic cell-matrix interactions which rely in part on receptors for collagen, one of the most abundant proteins of granulation tissue (Longaker and Adzick, 1991). Fibroblasts that migrate into the wound space deposit large quantities of ECM components, mainly collagen. The various types of collagen are believed to have profound effects on cells within the matrix and can influence cell differentiation and protein synthesis. For example phenotypic alterations of epithelial cells (Sugrue and Hay, 1981) as well as the expression of procollagenase in fibroblasts (Unemori and Werb, 1983) can be induced by collagen in vitro. In the context of my research focus, during wound healing, phenotypic modulation of fibroblasts can involve the generation of cells which show morphological and functional features of smooth muscle cell differentiation (Gabbiani et al., 1971).
structures and are composed of focal accumulations of cytoplasmic microfilaments and several ECM components, mainly collagen and fibronectin (Singer et al., 1984). Fibronexus structures are associated with the reorganization of the cytoskeleton in cell spreading and involve the formation of actin bundles including stress fibers. The force generated by intracellular tension is believed to be transmitted to the ECM through these structures (Ryan et al., 1974). Further, at least in part, cell-matrix links appear to control cell differentiation. For example endothelial cells can be switched between growth and differentiation based on their ability to adhere to the underlying substratum in vitro (Ingber, 1997). Therefore, it could be considered that cell-stroma interactions in granulation tissue induce fibroblast phenotype changes which in turn may orient the cell towards matrix remodeling. Collectively, the critical review of the data described above lead me to the conclusion that variations of intracellular and extracellular tension and forces can markedly influence cell differentiation, including the possible regulation of cytoskeletal genes. This regulation is clearly dependent on cell adhesion mechanisms. As there are several classes of cell-matrix and cell-cell receptors which are involved in cell adhesion and matrix remodeling, I will describe next one of the most important of these family of receptors, the integrin family.

2.2.2. Integrins

These heterodimeric proteins play an important role in the interactions between migrating fibroblasts and their substrates (Hynes, 1987). They are the major cell surface receptors for collagen and play an important role both in development and in the remodeling of connective and epithelial tissues in adult organisms. Integrins are comprised of α and β transmembrane glycoproteins (Hynes, 1987). This diverse superfamily consists of 8 known α and 14 known β subunits, many of which are expressed on a wide variety of
head, a membrane-spanning segment and a cytoplasmic domain which interacts with cytoskeletal proteins (e.g. talin, α-actinin) and perhaps other cytoplasmic domains (Burridge et al., 1988).

Signals can be transmitted through integrins in two directions. Inside-out signaling regulation mediates variation in the affinity and conformation of the receptor from inside the cell. For example in platelets, the cytoplasmic domain of the fibrinogen receptor (αIIbβ3) can control the binding affinity of the extracellular domain (O’Toole et al., 1991). For outside-in signaling, ligand occupation of receptor triggers intracellular events. Examples include integrin-mediated tissue remodeling by enzymatic activity (Werb et al., 1989) and by physical remodeling of collagen (Bell et al., 1979). Further, receptor-ligand interactions may lead to changes in the conformation of the cytoplasmic domain of the receptor which in turn leads to cytoskeletal reorganization (Burridge et al., 1988).

Among the different subgroups in this family, some integrins share a common β1 subunit. These integrins are known to mediate cell adhesion to ECM proteins (e.g. collagen, laminin, fibronectin), and also play an important role as mediators of migration, proliferation and differentiation (Hynes, 1987). The β1 family of integrins includes 6 members (Hemler, 1988) from which α1β1, α2β1, α3β1 are known to interact with collagen (Wayner and Carter, 1987; Belkein et al., 1990). α2β1 is believed to be the major collagen receptor on fibroblasts and is selectively up-regulated when fibroblasts are cultured in 3-D collagen matrices, suggesting a critical role in collagen remodeling (Klein et al., 1991). The α2β1 has also been shown to be involved in the migration of tumor cells within collagenous matrices (Yamada et al., 1990), and during tumor progression in human melanoma (Klein et al., 1991). Schiro et al. (1991) showed the involvement of the α2β1 integrin in the reorganization and contraction of hydrated collagen matrices using dermal fibroblasts. Cells
gels, but after transfection with α₂ cDNA they acquired the ability to remodel collagen. Another important finding in this paper was the central role of the cytoplasmic domain of the α₂ chain in the process of remodeling. Notably, the ability of the cells to bind to collagen does not predict their capacity to contract gels.

2.2.3. Fibroblast-Collagen Interactions and the Role of the Cytoskeleton in Matrix Reorganization

Fibroblasts are attracted into the wound space in part by fibronectin (Postlethwaite et al. 1981), PDGF (Seppa et al. 1982), and TGF-β (Postlethwaite et al. 1987). Immediately after migration they do not synthesize large quantities of type I collagen nor do they contain organized bundles of actin (Welch et al 1990). Throughout the granulation tissue migrating fibroblasts are capable of reorganizing connective tissue fibers, particularly collagen (Ehrlich et al., 1988). However, based on the theory of myofibroblast contraction, the force required for matrix remodeling during wound healing is mediated by specialized fibroblasts rich in filamentous actin (i.e. myofibroblasts). These cells are aligned within the wound along lines of contraction (Skalli and Gabbiani, 1988) and their appearance is often synchronous with the active stage of wound contraction (Welch et al., 1990). Upon entering the wound, myofibroblasts begin to synthesize large quantities of extracellular components such as collagen, fibronectin and other adhesion molecules, partly in response to TGF-β (Sporn et al., 1987). These adhesion molecules promote the formation of adhesion complexes and cell spreading. Since these phenotypically altered fibroblasts are suggested to play a central role in wound healing, in the next section I will review their differentiation and some of their characteristic features.
In recent years more attention has been paid to the functional differences between fibroblasts in spite of their relatively unified morphology. Fibroblast differentiation is difficult to study without well-defined markers as indicators of phenotypic modulations. Currently there are relatively few stable markers. However recent studies have suggested that the evaluation of cytoskeletal proteins, in particular actin isoforms, could be useful in this regard (Sappino et al., 1994).

Fibroblasts express several muscle-like differentiation features under certain physiologic and pathologic conditions in which there is tissue remodeling and fibrosis. For the first time in 1971, Gabbiani and co-workers reported the presence of modified fibroblasts in granulation tissue, with structural and biochemical properties intermediate between those of fibroblasts and smooth muscle cells. Myofibroblasts have a possible role in the contraction of granulation tissue during wound healing and in response to vasoconstrictors such as adrenaline (Gabbiani et al., 1972). The important ultrastructural and biochemical features of myofibroblasts are: 1) a well-developed actin microfilament system (Gabbiani, 1971) similar to smooth muscle cells (Chamley-Campbell et al., 1979) and cultured fibroblasts (Gabbiani et al., 1981); 2) numerous tight and gap junctions that connect them to each other (Gabbiani et al., 1978); and 3) fibronexus structures (Singer et al., 1984).

Several cytoskeletal markers has been studied as differentiation markers for these cells including vimentin, desmin, α-Smooth Muscle Actin (α-SMA) and smooth muscle myosin. Among them α-SMA is most commonly expressed by myofibroblasts (Sappino et al., 1990). Fibroblastic cells in granulation tissue were thought to express only vimentin (Skalli and Gabbiani, 1988) but investigations of healing wounds in rats demonstrated that a
The cellular origin of myofibroblasts is poorly understood but at least 3 precursor cell types have been proposed including fibroblasts, pericytes and smooth muscle cells. However the heterogeneity in expression of cytoskeletal proteins among these cells has made lineage studies very complicated (Schmitt-Graff et al. 1994). It has also been proposed that myofibroblasts in pathologic conditions may differentiate from local specialized cells, such as the interstitial cells in the lung septa (Kapanci et al., 1990) and the perisinusoidal cells in the liver (Schmitt-Graff et al., 1991). Whether myofibroblasts are a product of recruitment and proliferation of a subpopulation of the cells that constitutively express \( \alpha \)-SMA or whether local stimuli are capable of modulating myofibroblastic differentiation from resident stromal cells remains to be determined. The transient expression of \( \alpha \)-SMA during wound healing supports the second possibility (Darby et al., 1990).

Myofibroblasts have been described in numerous organs under normal situations such as in the pulmonary septa (Kapanci-Gabbiani 1974), ovary (O’shea 1970), and periodontal ligament (Beertsen 1974). A common feature of these diverse organs is their rapid rate of tissue remodeling either continuously or periodically. These data tend to provide further support for the role of myofibroblasts in matrix remodeling (Sappino et al. 1990). In pathologic conditions, \( \alpha \)-SMA expressing fibroblasts are present transiently during wound healing (Darby et al., 1990) and permanently in clinical disorders associated with prominent tissue retraction and remodeling such as hypertrophic scars, liver cirrhosis, pulmonary fibrosis and stromal reaction to the invasion of epithelial tumors such as breast cancer (Sappino et al., 1990).
muscle cells, however little information is presently available with respect to phenotypic changes in fibroblasts. Several factors such as the differentiation state, pathologic situations or culture conditions are known to change the expression pattern of actin isoforms in arterial smooth muscle cells. For example, α-SMA becomes prominent in smooth muscle cells as the cells differentiate (Kocher et al., 1985) whereas in pathologic situations such as arterial intimal thickening after endothelial injury and human atheromatosis (Gabbiani et al., 1984) there is decreased α-SMA expression and increased β-actin expression in smooth muscle cells. Culture conditions can also influence the expression of α-SMA in smooth muscle cells, since it has been shown that replicating smooth muscle cells express low levels of α-SMA and the expression increases as the cells reach confluence (Skalli et al., 1986). All of these results indicate that α-SMA expression in smooth muscle cells is modulated during various experimental and pathologic conditions but the mechanisms underlying these changes are unknown.

It has been proposed that the development of intracellular tension plays an important role in phenotypic modulations of fibroblasts but the mechanism is not clear. When fibroblasts are cultured on rigid substrates or in granulation tissue during wound contraction, they acquire the features of more differentiated smooth muscle cells including expression of α-SMA. Several studies point to α-SMA as a contractile isoform of actin. The increased expression of α-SMA evidently occurs in response to increased intracellular tension (e.g. aortic endothelial cells of hypertensive animals (Gabbiani et al., 1975), whereas reduced expression of α-SMA is associated with the decreasing contractile ability of smooth muscle cells such as in human atheromatous plaques (Gabbiani et al., 1984). As mentioned earlier, one of the characteristic features of myofibroblasts is the development of several types of cell-matrix linkages. A direct relationship between α-SMA expression and the formation of
Previous studies have also shown that the appearance of α-SMA is concurrent with the formation of focal contacts in epithelial cells and fibroblasts of granulation tissue. Perhaps the α-SMA isoform serves as a signal for wound contraction (Horiba et al. 1994). Taken together, these data imply that α-SMA may play a central role in contraction of the granulation tissue which is a vital aspect of wound closure. Therefore, factors capable of modulating myofibroblast differentiation and α-SMA expression are of great importance in the repair process.

3. REGULATION OF α-SMA EXPRESSION

In recent years great emphasis has been placed on the study of growth factors, cytokines and extracellular matrix components that can influence the growth and differentiation of stromal cells and their subsequent participation in wound repair. In this section I will review some of the factors that might contribute to the phenotypic modulation of fibroblasts and to the expression of α-SMA. It should be also pointed out here that the α-SMA promoter is different from the β-actin promoter and has different consensus sequences than other actin promoters and is apparently under separate transcriptional control (Hautmann et al., 1997).

3.1. Regulatory Factors

3.1.1. Extracellular Matrix Components

There is a substantial body of evidence to show that the extracellular matrix not only provides a structural support for cells but also plays a pivotal role in signal transduction, cell growth and differentiation (Juliano and Haskill, 1993). The development of mechanical stress in fibroblast-populated collagen gels has been suggested to be a determinant of
The role of the ECM in the regulation of TGF-β gene expression has also been demonstrated, since for example the presence of a functional basement membrane reduces the expression of TGF-β by epithelial cells (Streuli et al., 1993). Amongst ECM components, heparin can up-regulate α-SMA expression in smooth muscle cells (Desmouliere et al., 1991) and in cultured fibroblasts (Desmouliere et al., 1992a) and the mechanism appears to be selection of a subpopulation of α-SMA expressing cells rather than by phenotypic modulation of existing cells. Numerous growth factors and cytokines have been studied for their regulatory role in α-SMA expression and I will outline some of these below.

3.1.2. Growth Factors and Cytokines

PDGF is a well-recognized mitogenic and chemotactic factor for mesenchymal cells including smooth muscle cells and fibroblasts. It stimulates fibroblasts to produce both collagen and collagenase (Sappino et al., 1990). Its essential role in wound repair is well-established since it is released from platelets, monocytes/macrophages and endothelial cells during early stages of tissue repair (Sporn and Roberts, 1986). PDGF appears to have diverse effects on the expression of cytoskeletal differentiation markers in fibroblasts and smooth muscle cells. The correlation between PDGF gene expression by lung carcinoma cell lines and stromal reactions to tumor cells indicates that PDGF may be produced during conditions in which fibroblasts express smooth muscle cell differentiation markers including α-SMA (Bergh et al., 1988). However PDGF appears to reduce the expression of α-SMA in cultured smooth muscle cells (Corjay et al., 1989). Further, PDGF fails to induce α-SMA expression in cultured skin derived fibroblasts (Sappino et al., 1990). Fibroblast Growth Factor is released by macrophages and endothelial cells and is known to be a potent mitogen for fibroblasts and endothelial cells. It also stimulates the accumulation of collagen
by FGF, but this was suggested to be due to the enhanced proliferative activity promoted by FGF (Schmitt-Graff et al., 1994).

Granulocyte Macrophage-Colony Stimulatory Factor (GM-CSF) is known essentially for its stimulating effect on growth and differentiation of hematopoietic cells. Further, GM-CSF acts as a proliferative stimulus for some mesenchymal cells including endothelial cells and marrow fibroblasts (Dedhar et al., 1988). GM-CSF has been demonstrated to induce formation of granulation tissue rich in α-SMA in rat dermis (Rubbia-Brandt et al., 1991). In vitro experiments indicate that GM-CSF does not directly induce up-regulation of α-SMA and that its regulatory function is probably mediated through macrophages (Schmitt-Graff et al., 1994). Moreover, the development of fibrotic nodules that are spatially associated with macrophages and rich in cells expressing α-SMA was demonstrated in transgenic mice over-expressing GM-CSF (Lang et al., 1987).

Endothelin is a potent vasopressor that was originally isolated from cultured porcine endothelial cells (Yanagisawa et al., 1988). Stimulation of vascular smooth muscle cells by growth factors (e.g. TGF-β and PDGF) has been shown to increase endothelin-1 release and that endothelin-1 induces the expression of α-SMA in cultured smooth muscle cells (Hahn et al., 1992).

γ-IFN is released by helper T-lymphocytes and this cytokine inhibits the proliferation and expression of α-SMA in cultured smooth muscle cells (Hansson et al., 1989) and fibroblasts (Desmouliere et al., 1992b). Further, its inhibitory effect on collagen production by fibroblasts suggests the anti-fibrotic activity of this cytokine in vivo (Grandstein et al., 1990).
TGF-β a cytokine that induces up-regulation of α-SMA in human dermal fibroblasts (Sappino et al., 1990). Further, accumulation of α-SMA expressing fibroblasts is observed around clusters of macrophages releasing high levels of TGF-β in bleomycin-induced fibrotic lung (Khalil et al., 1989). Desmouliere and co-workers (1993) have suggested that TGF-β is the only substance that can induce the expression of α-SMA in quiescent cultured fibroblasts.

In summary, it is believed that phenotypic modulation of fibroblasts and the expression of α-SMA involves the complex interaction of several factors and there is no single factor acting alone that can induce phenotypic changes in fibroblasts in vivo. This also underscores the role of inflammatory cells in granulation tissue (such as macrophages) that can produce a wide range of growth factors including TGF-β in fibroblastic differentiation.

TGF-β is a well-known regulator of extracellular matrix formation and cell-matrix interactions. Further, the previously described effects of TGF-β on α-SMA expression (Desmouliere et al., 1993) suggest that it plays an important role in fibroblast differentiation. Therefore in the next section I will review the diverse activities of this growth factor, in particular those related to tissue repair and the expression of α-SMA.

3.2. TGF-β

TGF-β is the prototype of a large family of polypeptides involved in the regulation of cell growth and differentiation (Massague, 1987). This 25 kD disulfide-linked dimer was originally characterized by its ability to induce anchorage-independent growth in normal fibroblasts (Roberts et al., 1981). There are three major isoforms of TGF-β in mammals (TGF-β₁, β₂, β₃ ) which are homodimers of β₁, β₂ and β₃ subunits respectively (Attisano et
main TGF-β-binding components (Massague et al., 1994). TGF-β is released by almost all cell types and the highest concentrations of TGF-β in the body is found in platelets (Roberts et al., 1986; Sporn et al., 1986). Fresh human platelets contain 200-300 mg/ml TGF-β (Wakefield et al., 1988) which is in a biologically inactive form and is unable to bind to TGF-β receptors (Wakefield et al., 1987). The exact mechanism of activation in vivo is not clear but it is believed to be either as a result of exposure to acidic environments such as is found in healing wounds or by the action of serine proteases involved in the clotting cascade (Wakefield et al., 1988). Some serum proteins such as α2-macroglobulin may lead to masking of TGF-β (O’Conner and Wakefield, 1987). The binding of TGF-β to ECM components including collagen IV (Paralkar et al., 1991) and proteoglycans (Andres et al., 1984) may serve as a reservoir for active TGF-β or a way to sequester this factor in an active or inactive form and so prevent its wide spread diffusion. Since receptors for TGF-β are expressed on a wide variety of cell types, activation of TGF-β is likely a critical regulatory step in TGF-β function (Wakefield et al., 1988). In laboratory experiments the latent form has been activated generally by transient acidification, probably by the disruption of a non-covalent complex (Lawrence et al., 1985).

TGF-β has diverse effects on different cell types, such as stimulation of proliferation in osteoblasts (Robey et al., 1987) and growth inhibition of epithelial cells (Masui et al., 1986) and many mesenchymal cells including T and B lymphocytes, endothelial cells and embryonic fibroblasts (Sporn et al., 1987). TGF-β is chemotactic for fibroblasts and stimulates matrix formation while inhibiting its degradation (Postlethwaite et al., 1987), emphasizing its critical role in embryogenesis and tissue repair. The release of TGF-β from degranulated platelets, activated macrophages and T-lymphocytes during early tissue responses to injury highlights its involvement in wound healing events (Sporn et al., 1987).
Indeed the presence of TGF-β in experimental rat wound chambers has been shown at different stages of wound healing with a peak at day 7 after wounding (Cromack et al., 1987). However in another study TGF-β was not detected in humanchronic pressure wounds (Cooper et al., 1994). TGF-β can promote wound healing (Brown et al., 1988; Beck et al., 1990) and reverse the negative effects of age or glucocorticoids on wound healing (Beck et al., 1993). The well-known role of TGF-β in extracellular matrix accumulation suggests the involvement of this growth factor in fibrotic diseases of the organs as well as wound scarring. In this context the major difference between fetal and adult wound healing is that fetal wounds heal without scarring and that only after birth does the healing of a wound generate a scar. Recently a relationship between scarless fetal wound healing and the greatly reduced or absence of a TGF-β response to wounding has been demonstrated (Whitby and Ferguson, 1991).

As TGF-β is believed to be capable of influencing different stages of wound healing, I will focus on the role of TGF-β in wound contraction by reviewing the its ability to modulate the expression of the contractile actin isoform, α-SMA. TGF-β up-regulates α-SMA expression in fibroblasts and endothelial cells (Desmouliere et al., 1993, Bjorkenid et al., 1991). The presence of a subpopulation of α-SMA positive cells in populations of cultured fibroblasts even after cloning suggests that α-SMA is expressed when serum (and therefore TGF-β) are present (Desmouliere et al., 1992). Moreover TGF-β is known to be the only substance capable of stimulating α-SMA expression in cultured quiescent fibroblastic cells (Desmouliere et al., 1993). Whereas a recent study has shown that the absence of cell-cell contact is required for TGF-β induction of α-SMA in fibroblasts (Masur et al., 1996). Several studies have established a relationship between TGF-β and pathologic settings associated with fibrosis in which α-SMA expressing fibroblasts are present. Khalil et al. (1989) have shown that in bleomycin-induced pulmonary fibrosis, an accumulation of
high levels of TGF-β. Further, a marked up-regulation of α-SMA expression was associated with experimental hydronephrosis in rats which is characterized by enhanced macrophage infiltration as well as increased TGF-β1 gene expression (Diamond et al., 1995). Taken together these data suggest that TGF-β may regulate ECM remodeling by affecting the cellular content of α-SMA.

One of the important roles of TGF-β in tissue repair is its stimulatory effect on matrix synthesis which occurs as a consequence of TGF-β induced up-regulation of several ECM components including collagens type I, II, III, IV, V, osteopontin, osteonectin, elastin, proteoglycans (Roberts et al., 1990). At the same time it inhibits matrix degradation by both the down-regulation of proteases such as collagenase, and up-regulation of the protease inhibitors such as tissue inhibitor of metalloproteinase (Roberts et al., 1992). In parallel with this finding, the increased levels of TGF-β in vivo could result in pathologic situations characterized by excessive matrix formation such as pulmonary fibrosis, glomerulonephritis and proliferative vitreoretinopathy (Roberts et al., 1992). In an experimental model of glomerulonephritis it has been shown that systemic administration of anti-TGF-β antibodies suppresses the symptoms of the disease (Border et al., 1990). TGF-β also regulates cell-matrix interactions by regulating the expression of integrins, important cell adhesion molecules. TGF-β up-regulates the expression of all members of the β1 integrin family. This family is known to interact with several ECM components such as collagen, fibronectin and laminin, in a cell-specific manner (Heino et al., 1989).

Briefly, TGF-β is known to up-regulate the expression of α-SMA in endothelial cells and fibroblasts. Further, the development of mechanical stress in tissues may play a key role in cell responsiveness to other growth factors including PDGF, FGF, TGF-β and IL-1 (Nakagawa et al., 1989a). What is currently unknown is if tension affects TGF-β
responsiveness to TGF-β is regulated.

4. IN VITRO MODELS OF CONTRACTION

Fibroblasts in vivo reside within a collagenous stroma and are capable of physically reorganizing their substrate. The nuances of the extracellular matrix remodeling processes were not appreciated from studies of cells in classic monolayer cultures because of limitations imposed by the nature of the substrate and by the lack of a 3-dimensional matrix. Consequently an important innovation of in vitro models of collagen remodeling was the ability to culture fibroblasts in a 3-dimensional substrate.

4.1. Fibroblast Populated Collagen Lattices (FPCL) as an In Vitro Model for Wound Contraction

Elsdale and Bard (1972) plated fibroblasts in 3-dimensional collagen lattices, but they did not report lattice contraction or formation of a tissue-like structure as a consequence. In 1979, Bell and co-workers used the contraction of a hydrated collagen lattice as an assay for measuring fibroblast function and reported the formation of a tissue-like fabric that could be used as a model in wound healing studies. Bellows et al. (1981) plated fibroblast-like cells from periodontal ligament in collagen lattices and reported that these cells establish numerous intercellular contacts and form a 3-dimensional network within the substrate. Tomasek et al. (1984) demonstrated that fibroblasts in collagen gels have morphological features similar to fibroblasts in vivo, suggesting that the collagen gel environment mimics the in vivo situation more closely than monolayer culture.

There are three common types of collagen gel models: floating gels, anchored gels and stress-relaxed gels. Each model has different mechanical features. In floating gels, the
decrease in all dimensions of the gel. Consequently, tension in this system is distributed isotropically. Anchored gels are attached to the underlying surface and as fibroblasts reorganize them, there is only a reduction in gel height; there is no change in gel diameter. Tension is distributed anisotropically and fibroblasts “contract” under isometric condition. Based on morphologic and biosynthetic data presented by Grinnell et al. (1991), fibroblasts in floating gels provide a model of resting dermis while fibroblasts in anchored gels provide a model of active granulation tissue that is undergoing contraction against an attached, relatively inflexible underlying bed. Stress-relaxed gels are anchored gels that are released from the surface after development of tension in the substrate and may provide a model for the transition from granulation tissue to dermis. The contraction in this system occurs very quickly. Along with the contraction of fibroblasts there is collapse of actin filament bundles (Mochitate et al., 1991; Tomasek et al., 1992) and the contraction resembles smooth muscle-like contraction rather than tractional remodeling. Below I will briefly review the morphologic and biochemical differences between fibroblasts in floating vs. anchored gels.

In floating gels fibroblasts develop a stellate morphology with long processes and a well-developed cytoskeletal meshwork (Bell et al., 1979; Bellows et al., 1981). However in anchored gels, cells become bipolar and orient along the lines of tension (Bellows et al., 1981). The cells also develop stress fibers and fibronexus junctions and resemble myofibroblasts (Farsi and Aubin, 1984; Mochitate et al., 1991; Tomasek et al., 1992). The study of fibroblasts in anchored and floating gels has revealed remarkable differences in cell responsiveness to growth factors between the two systems. In floating gels there is a significant decrease in cellular DNA synthesis (Nishiyama et al., 1989; Nakagawa et al., 1989a), cells become arrested in G₀ (Kono et al., 1990) and may begin to die (Nakagawa et al., 1989a and 1989b). In anchored gels cells continue to synthesize DNA and proliferate.
differences in the mechanical reorganization between the two models, since subjecting fibroblasts in floating gels to external stresses increases cell proliferation (Jain et al., 1990). In general, cells in floating gels seem to be unresponsive to different growth factors including FGF, PDGF, TGF-β and IL-1, and none of these growth factors can restore the rate of DNA synthesis to levels observed in anchored gels (Nakagawa et al., 1989a). Recent studies show that PDGF receptors on fibroblasts in floating gels become unresponsive to PDGF (Lin and Grinnell, 1993). The growth inhibition of fibroblasts in floating gels may be independent of the extent of gel contraction and is influenced more by cell shape and ECM reorganization (Nakagawa et al., 1989a).

The reorganization of a fibroblast-populated collagen gel during contraction, regardless of its type is denoted tractional remodeling (Harris et al., 1981). Tractional remodeling is believed to occur as a result of adhesive cell-matrix interactions and depends on an intact cytoskeleton. The outcome of this remodeling however, is significantly different between the two types of models. In floating gels the end result of contraction is a mechanically relaxed tissue whereas in anchored gels contraction results in a stressed tissue.

As described earlier, some of the members of the β1 integrin family (i.e. α1β1, α2β1, α3β1) are known to mediate fibroblast interaction with collagen fibers (Wayner et al., 1987; Belkin et al., 1990; Kirchofer et al., 1990). Amongst these receptors α2β1 plays a pivotal role in remodeling of collagen type I gels (Schiro et al., 1991; Klein et al., 1991).

The force required for contraction is thought to be generated by actin-myosin interaction in fibroblasts and depends on an intact cytoskeleton (Bell et al., 1979; Guidry et al., 1985), myosin light chain kinase activity (Ehrlich et al., 1991) and cyclic AMP as an inhibitory regulator of cell contraction (Van Bockxmeer et al., 1984). It has been shown that
establishment of tension in anchored collagen gels since colcemid and cytochalasin D treatment abrogates the ability of the cells to develop tension (Bellows et al., 1982). Also, Tomasek et al., (1984) have suggested that the actin cytoskeleton acts along with microtubules to bring about cell elongation in anchored gels.

Several factors have been shown to affect the rate of contraction in 3-dimensional collagen gels which I will review here. The contraction rate is directly proportional to cell density and inversely proportional to gel concentration (Bell et al., 1979). Also, a direct relationship between passage number and the rate of a fibroblasts-populated collagen lattice (FPCL) contraction has been demonstrated (Murphy and Daniel, 1987). Fibroblasts from different tissues such as skin, tendon and cartilage demonstrate distinct contraction profiles. Further, different sub-populations of fibroblasts can be distinguished in the same tissue based on their contraction profiles (Schafer et al., 1990; Kasugai et al., 1990; Eastwood et al., 1996). Previous work has shown that intra-oral fibroblasts exhibit a more fetal-like phenotype than extra-oral fibroblasts (Sloan, 1991; Irwin et al., 1994), and that this difference is reflected in their ability to remodel the ECM. Fibroblasts from oral mucosa more rapidly contract collagen gels than dermal fibroblasts (Stephens et al., 1996) suggesting that fibroblasts from tissues with high rates of ECM remodeling in vivo are more efficient in contracting collagen lattices than tissues with slower rates of remodeling (i.e. dermal fibroblasts). However, studies by Ehrlich et al. (1988) have shown that fibroblasts from contracting scars have an equal rate of collagen gel contraction as fibroblasts from normal tissue. In contrast, lattice contraction is faster in a FPCL made from collagen from a contracting scar compared to one made with normal dermal collagen. Hence, the collagen composition of a FPCL can apparently control the rate of contraction.
indicating that the ability of the cells to contract collagen matrices can be regulated by extracellular factors. Numerous growth factors have been studied for their ability to affect FPCL contraction. One of these factors, PDGF stimulates collagen matrix contraction (Clark et al., 1984; Gullburg et al., 1990; Tingstrom et al., 1992). There is contradictory evidence in the literature concerning the effect of TGF-\(\beta\) on gel contraction. A number of studies have shown the stimulatory effect of TGF-\(\beta\) on FPCL contraction (Montesano et al., 1988; Fukamizu et al., 1990) but these reports also indicate that PDGF has a more rapid and pronounced effect on fibroblast-mediated gel contraction than TGF-\(\beta\), suggesting an indirect role for TGF-\(\beta\) which may be mediated through PDGF (Montesano et al., 1988). However a more recent investigation reports that the mechanism of TGF-\(\beta\)-induced collagen contraction is independent of PDGF action (Tingstrom et al., 1992). In another study by Gullberg et al., (1990) there was no effect of TGF-\(\beta\) on the remodeling of collagen gels. FGF at low doses (0.1 to 1 ng/ml) exhibits moderately stimulatory effects, whereas high doses (<10 ng/ml) inhibits basal contraction of collagen gels populated with granulation tissue fibroblasts (Finesmith et al., 1990). EGF has also been shown to inhibit contraction of collagen gels by fetal fibroblasts (Piscatelli et al., 1994). Another inhibitory factor is \(\gamma\)-Interferon (Gillery et al., 1992). The mechanisms by which these factors modulate the contraction of collagen matrices are unknown but protein kinase C is suggested to play a role as a second messenger (Guidry et al., 1992).

In summary, a large body of information suggests that there are intrinsic differences between fibroblasts from diverse origins and at different stages of life. At the same time, local stimuli such as polypeptide growth factors may operate at the site of tissue repair to alter cell phenotype and influence tissue remodeling. However, another line of studies indicates that fibroblasts in granulation tissue are not different from normal tissue, thereby questioning the involvement of specialized fibroblasts known as myofibroblasts in wound contraction, while emphasizing the role of dynamic cell-matrix interactions in producing the
Although these two concepts are considered separately, they are not exclusive and recently these two theoretical approaches have been combined by Grinnell (1994). He has developed a new hypothesis in which mechanical traction forces in vivo may stimulate myofibroblast differentiation by cell-matrix interactions. Based on this recent theory, wound contraction begins with the migration of fibroblasts into the wound space. The resistance of granulation tissue to contraction leads to a switch of differentiation of normal fibroblasts into myofibroblasts which further promotes contraction. I believe that this theory rather neatly brings together two apparently contradictory notions into a unified model which better explains a large body of experimental data than either single theory.

4.2. Effect of TGF-β and α-SMA Expression on Gel Contraction

It has been shown that the activity of serum in contraction of FPCLs can be replaced or promoted by TGF-β (Montesano and Orci, 1988). Moreover, comparisons of fibroblasts from wounds at different stages of repair and normal skin have revealed the greater ability of cells from older granulation tissue to contract collagen matrices compared to cells from younger granulation tissue or normal skin. Further, the promoting effect of TGF-β on contractility was more prominent in the cells with greater basal contractile ability (Finesmith et al., 1990). Fibroblasts from fetal granulation tissue become primed for collagen contraction at an earlier stage in the wound healing process than in the adult and TGF-β stimulates contraction of collagen matrices by fetal fibroblasts (Piscatelli et al., 1994). This correlates with the observation that TGF-β converts fetal healing to an adult type of healing which is accompanied by the induction of marked fibrosis (Lin et al., 1995). Fibroblasts derived from bleomycin-induced fibrotic lungs show a greater ability in mediating collagen gel contraction as well as higher expression of TGF-β gene compared to fibroblasts from normal lungs (Zhang et al., 1996). Furthermore, bleomycin-induced lung fibrosis is accompanied by phenotypic changes in lung fibroblasts characterized by smooth muscle-like differentiation and the expression of α-SMA (Shurch et al., 1992). In a study
contraction by gingival fibroblasts showed the dependence of collagen remodeling on the expression of α-SMA and suggested that α-SMA might be a marker of a contractile phenotype. This is in agreement with the finding that the transition from fetal (scarless) healing to healing with scarring which occurs late in gestation, corresponds with the appearance of α-SMA expressing myofibroblasts in granulation tissue (Estes et al., 1994). Taken together these findings suggest that although all fibroblasts possess contractile ability, α-SMA expressing fibroblasts are even more contractile and exhibit greater capacity for matrix remodeling.

The regulatory factors that account for the qualitative differences between different types of fibroblasts are poorly understood and so are the changes that occur in fibroblasts under different situations such as wound healing. Several growth factors including TGF-β have been suggested to be involved in altering the phenotypic features of fibroblasts but the downstream signaling mechanisms are unknown. Moreover, it has been implied that the mechanical changes that occur in tissue under certain conditions such as wound healing may alter fibroblast responsiveness to growth factors. How this alteration happens remains to be explored, but some suggestions have been made, including the alteration in the number of the receptors for growth factors, or selective changes in signal transduction machinery, or in the cytoskeleton.
Current evidence suggests that fibroblasts undergo phenotypic modulation during several physiologic and pathologic conditions such as wound healing and fibrocontractive diseases (Schurch et al., 1992). The phenotypically altered fibroblasts develop cytoskeletal features similar to those of smooth muscle cells, including the expression of α-SMA (a contractile isoform of actin) and hence have been denoted as myofibroblasts (Gabbiani et al., 1971). The myofibroblast is suggested to contribute to the process of wound contraction, as well as contractile phenomena observed during fibrotic diseases. However, little is known about the mechanisms responsible for phenotypic modulation of fibroblasts and the expression of α-SMA. Several types of growth factors and cytokines that are locally released from inflammatory and stromal cells at wound sites, as well as ECM components, have been examined to discover their role in fibroblastic differentiation. A likely candidate for regulation of α-SMA is TGF-β (Desmouliere et al., 1993), a potent regulator of ECM remodeling. This regulation may be achieved in part by modulation of cell-matrix interactions.

The development of mechanical stress in tissue can be a determinant of cell responsiveness to growth factors (Nakagawa et al., 1989a and 1989b). Mechanical stress could also regulate myofibroblastic differentiation under certain conditions such as the repair processes in which fibroblasts are involved. Indeed fibroblasts are likely exposed to different levels of both mechanical stress and TGF-β. Therefore in this study I have focused on the effect of TGF-β on α-SMA expression by fibroblasts cultured in collagen matrices of 3 different levels of mechanical deformability.

For these experiments I have used 3 tissue culture models with different mechanical features, including monolayer cultures, anchored gels and floating gels. Further, as serum contains several growth factors and cytokines that can influence the expression of α-SMA
inactivate it, I have evaluated the effects of TGF-\(\beta_1\) in both plus and minus serum conditions.

The experiments used in this study were aimed to test the hypothesis that TGF-\(\beta_1\)-induced regulation of \(\alpha\)-SMA expression in human gingival fibroblasts is dependent on the deformability of the substrate and on the development of intracellular tension.

To address this hypothesis my objectives were to:

1. Compare the effect of TGF-\(\beta_1\) on cell morphology, development of stress fibers and expression of \(\alpha\)-SMA in monolayer cultures, anchored and floating collagen gels.
2. Assess TGF-\(\beta_1\)-induced changes in contraction of anchored and floating collagen gel matrices.
4. Determine the importance of the \(\alpha_2\beta_1\) integrin on TGF-\(\beta_1\) induction of \(\alpha\)-SMA.

In a separate series of experiments, designed to assess whether substrate type may also affect levels of TGF-\(\beta_1\), I compared the effect of the 3 different models on the synthesis of TGF-\(\beta_1\) by gingival fibroblasts.
Cell Cultures

Primary cultures of human gingival fibroblasts (HGFs) were obtained from biopsies of normal gingiva in patients aged between 10 to 16 years as described (McCulloch and Knowles, 1993). Tissues were collected in serum-free α-minimal essential medium (MEM; Faculty of Medicine, Toronto) containing 10% antibiotic solution (0.017% penicillin G [Ayerst Lab., Montreal, PQ], 0.01% gentamycin sulphate [Life Technologies Inc., Grand Island, NY], in α-MEM). Fragments of 1 mm³ were removed from the gingival tissue, and each biopsy was placed into a single well of a 24-well multi-chamber slide (Labtek, Naperville, IL) containing growth media consisting of 75% (v/v) α-MEM, 10% antibiotics (v/v) and 15% (v/v) fetal bovine serum (serum). Cultures were left undisturbed for 10 days at 37°C in a humidified atmosphere of 5% CO₂- 95% air. In order to expand the cultures, confluent cell monolayers were washed with phosphate buffered saline (PBS), detached with 0.01% Trypsin (Gibco Laboratories, NY), re-plated and grown to confluence first in T-25 flasks (Costar, Cambridge, MA) and then in T-75 flasks (Becton Dickson, Mississauga, ON). Cells were subcultured at a split ratio of 1:3 approximately every 3-5 days. Cells between passages 3 and 12 were used for all experiments.

3-Dimensional Hydrated Collagen Matrices

Collagen solutions were prepared as described (Bellows et al., 1981). Briefly, under sterile conditions, a collagen solution was prepared from 0.3 ml of 10× concentrated α-MEM, 0.3 ml of 0.26 M NaHCO₃ buffer, 0.3 ml serum, 0.12 ml of 0.1 M NaOH and 1.5 ml of Vitrogen 100 (Collagen Corp, Santa Clara, CA). A cell suspension of 4×10⁵ cells/ml in α-MEM ± Serum was added to the solution. Gel solutions were pipetted into tissue culture or non-tissue culture dishes in order to obtain anchored or
incubated at 37°C in 95% air and 5% CO₂ for 1 hour until polymerization had occurred and were then covered with growth medium.

Collagen-Coated Plates

Petri dishes without tissue culture treatment (Falcon; Becton Dickinson, Mississauga, ON) were incubated overnight at room temperature with Vitrogen 100 diluted in 0.01 N HCl at a ratio of 1:5 (dilution: 0.5 mg/ml). To block non-specific binding, plates were incubated with 1% (w/v) bovine serum albumin for 4 hrs at 4°C. Before plating the cells, dishes were rinsed with PBS to remove excess BSA.

α-Smooth Muscle Actin Expression in HGFs

Immunofluorescence staining of α- Smooth Muscle Actin (α-SMA) was conducted on monolayer cultures, anchored and floating collagen gels to evaluate the effect of Transforming Growth Factor-β₁ (TGF-β₁) on α-SMA expression and colocalization in stress fibers, as well as changes in cell morphology.

Monolayer Cultures:

Coverslips were placed in 24-well plates (Corning, New York) and coated with collagen. Cells in T-75 flasks were trypsinized (0.01% Trypsin) and re-suspended in α-MEM plus 15% serum at a concentration of 4×10⁵ cells/ml (final plating density of 7.5×10⁴ cells/cm²) which was kept constant for all of the experiments. Triplicate cultures were performed for each treatment. After 24 hours of incubation, medium was removed, cells were covered with α-MEM ± Serum alone or supplemented with 10 ng/ml Porcine TGF-β₁ (R&D Systems) and incubated for 3 days. At the end of the treatment cells were fixed in paraformaldehyde (2%) for 15 minutes at room temperature, followed by permeabilization with 0.3% Triton and thorough rinsing with PBS. To stain for α- SMA,
Sigma) for 1 hour at 37°C followed by a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (1:100; Sigma) for one hour at 37°C. Pre-immune mouse and goat sera were used to block non-specific staining prior to antibody incubation. All coverslips were counter-stained with 4-6-diamidino-2-phenyl-indole (DAPI; 1µg/ml; Sigma) for nucleus staining to facilitate microscopic localization of cells. Preparations of fixed cells were mounted with immuno-fluore mounting solution (ICN) and examined in a Leitz Orthoplan microscope (25× objective; Leica, Wetzlar, FRG) equipped with specific filters for rhodamine, fluorescein and DAPI.

**Collagen Gels:**

Samples (150 µl) of cell/collagen mixture (4×10^5 cells/ml) were pipetted into 35 mm tissue or non-tissue culture dishes (3 samples per dish, [Falcon]). After polymerization the gels were covered with α-MEM ± 15% serum for 24 hrs. The medium was removed and gels were covered with α-MEM ± 15% serum, with or without TGF-β1 for 3 days. Immunofluorescence staining for α-SMA was conducted as described for monolayer cultures. After mounting the gels, single cells were imaged on a confocal microscope (Leica CLSM, Leica, Heidelberg, Germany). For FITC, excitation was set at 488 nm and emission at 530 nm. Cells were imaged with a 40× oil immersion lens (N.A. 1.2), and sections were obtained by optical sections at a nominal thickness of 1 µm. In some samples these computer-generated images were then combined to reconstruct a composite image.

**Microfilament Rearrangement**

The effect of TGF-β1 on development and reorganization of stress fibers was investigated using affinity labelling of filamentous actin. Cells were plated on collagen coated coverslips in 24-well multi-chamber slides (7.5×10^4 cells/cm^2) and treated as
fixed for 15 minutes with paraformaldehyde and washed again with PBS. Filamentous (F)-actin was stained for 15 minutes with $5 \times 10^{-6}$ M tetra-methyl rhodamine isothiocyanate (TRITC)-Phalloidin (Sigma) in PBS containing 0.01% Nonidet. Cells were also stained with DAPI to facilitate cell counting. Finally the coverslips were washed, air-dried and mounted with an anti-fade reagent. The total cell fluorescence due to (TRITC)-Phalloidin was measured in a standardized area ($100 \mu m^2$) using a Leitz MVP-SP Spectrofluorimeter (Wetzlar, Germany) and a 25× Plan Apo Objective. Excitation was set at 530/20 nm and emission due to TRITC-phalloidin was set at 600/3 nm. The photomultiplier tube voltage was set to 599 V and the amplifier gain to 4×. The fluorescence of standardized background areas was subtracted from each sample measurement to correct for background and dark current. For each treatment, 10 randomly chosen areas were measured in each of 3 preparations. The value for each area was divided by the number of the cells and the average fluorescence intensity calculated for a single cell.

**Quantitative Analyses of α-SMA & α₂β₁ Integrin**

**α-Smooth Muscle Actin**

To quantify TGF-β₁-induced changes of α-SMA at the protein level, western blot analysis was performed on cell extracts prepared from monolayer and collagen-gel cultures.

**Monolayer Cultures:**

Cells were plated in 35 mm tissue culture or collagen-coated Petri dishes at the concentration of $7.5 \times 10^4$ cells/cm² and incubated with α-MEM ± 15% serum, in the presence or absence of TGF-β₁ as described above. Triplicate samples were assayed at 24
250 µl of 2× SDS sample buffer (1.52 g Tris Base, 20 ml glycerol, 2 g SDS, 2 ml 2-mercaptoethanol in 100 ml H₂O) or first detached with trypsin (0.01%) or EDTA (0.5 M), rinsed with Mg²⁺, Ca²⁺ free-PBS and the pelleted cells (collected by centrifugation at 14000 RPM for 5 minutes; Eppendorf Centrifuge 5402) dissolved in sample buffer. Content was assessed by the BioRad assay. Samples were boiled for 3 minutes at 95°C and equal amounts of protein were electrophoresed on a 10% gradient SDS gel. Separated proteins were transferred to nitrocellulose filters which were then incubated with mouse monoclonal antibody for α-SMA (1:1000; clone 1A4, Sigma). Blots were stripped and probed with a monoclonal antibody for β-Actin (clone AC-15) for comparison. The first antibody incubation was conducted at room temperature for 1 hour, followed by 3 washes 15 minutes each with PBS-tween. A second incubation with a goat anti-mouse antibody conjugated with HRP (1:1000) and ECL reagents (Amersham) was followed by 3 washes. Subsequently X-OMAT Kodak films were exposed to the blots and the density of the bands were scanned by Scan Analysis (Biosoft, Cambridge, U.K.).

To determine if there is leakage of α-SMA into the media following cell detachment, EDTA-detached cells were also analysed for α-SMA and β-Actin protein. (0.5 mM) was used as a non-proteolytic agent to detach the cells. Cell pellets were separated from the supernatants by centrifugation at 14000 RPM for 5 minutes. To eliminate the extra salt, cell supernatants were dialysed overnight in dialysis membranes (Spectra/Por 4) in 50 mM NH₄HCO₃ and then concentrated in Microcons (model 100) by centrifugation at 3000 g-force to obtain a final volume of 200 µl. Cell supernatants were probed for α-SMA and β-Actin protein as described above.
Cell/Collagen solutions were pipetted into 35 mm tissue culture or non-tissue culture dishes (1.5 ml per dish) and incubated under the same conditions described above. To harvest fibroblasts from the gels, each gel was rinsed thoroughly with Mg\(^{2+}\), Ca\(^{2+}\) free-PBS and incubated for 10 minutes at 37°C with 0.3 ml of 0.05% Trypsin/0.53mM EDTA solution, followed by 20-30 minutes incubation with 0.35 ml collagenase (5 mg/ml). After cells were dispersed completely, enzymatic activity was blocked by the addition of 0.05 ml FCS. Fibroblasts were collected by centrifugation at 14000 RPM for 10 minutes at 22°C and 0.05 ml of extraction buffer (2% Triton X-100, 160 mM KCl, 40 mm TRIS-HCl, 20 mM EGTA, 10 mM PMSF, 1 mM Leupeptin, 1 mM Benzamidine; Sigma, St. Louis, MO) was added to each sample and boiled for 3 minutes at 95°C. Immunoblotting for α-SMA and β-Actin was conducted for triplicate samples as described for monolayer cultures.

Expression of α\(_2\) & β\(_1\) integrin subunits

To investigate the effect of TGF-β\(_1\) on the expression of α\(_2\) and β\(_1\) integrin subunits, western blotting was performed on cell extracts prepared from monolayer cultures of fibroblasts on collagen-coated 60 mm plates. Twenty four hours after plating the cells, medium was replaced with α-MEM ± 10 ng/ml TGF-β\(_1\) and cells extracted after a further 12 or 24 hr of incubation. Blots were prepared as described above and were probed for expression of α\(_2\) and β\(_1\) using mouse monoclonal antibodies (clone P1H5; Calbiochem, Temescula, CA, and 4B4-RD1; Coulter, Burlington, ON, respectively). β-actin antibody was used for normalization of the protein loading and for comparisons.

Single Cell Analyses of α-SMA & α\(_2\)β\(_1\) Integrin Expression

Flow cytometric analyses of α-SMA and α\(_2\)β\(_1\) integrin were conducted to investigate the TGF-β\(_1\)-induced changes of the expression level of these proteins in single
plated on 100 mm collagen coated dishes; 24 hours later the media were replaced with α-MEM ± 10 ng/ml TGF-β1 and cultures were incubated for 3 days.

**α-Smooth Muscle Actin**

Single cell suspensions were prepared by trypsinization (0.01% trypsin). Cells were fixed with 3.7% formaldehyde, permeabilized in 0.02% Triton with PBS, stained for α-SMA as described (Arora and McCulloch, 1994). First and 2nd antibody dilutions were 1:30 and 1:75 respectively. Cells were washed and resuspended in Mg²⁺, Ca²⁺ free-PBS. Samples were analysed on a FACSTAR Plus flow cytometer (Becton-Dickinson, Mississauga, ON) with 488 nm excitation and 530/30 nm band pass filter for FITC-labeled antibodies. For all flow cytometry analyses at least 1×10⁴ cells were assessed in each sample and only cells with forward and orthogonal light scatter characteristics similar to whole, intact fibroblasts were included in the analysis by electronic gates previously established for fibroblasts.

**α₂ and β₁ integrin subunits**

All the procedures were similar to that employed for α-SMA assessment. Mouse monoclonal antibodies for α₂ (1:20 dilution, clone P1H5) and β₁ (5 µl/10⁶, clone 4B4-RD1) integrins were used.

**Northern Analysis**

Northern blots were performed on monolayer cultures to determine the effect of TGF-β₁ on levels of mRNA for α-SMA. Fibroblasts were plated on collagen-coated 150 mm Petri dishes (7.5×10⁴ cells/cm², [Falcon]). Twenty four hours later medium was removed and cells were covered with α-MEM ± 15% serum with or without 10 ng/ml TGF-β₁ and incubated for 3 days. Total RNA was isolated using the protocol used by Chomczynski et
isothiocyanate (5.3 M), sodium citrate (30 mM), 0.27% N-laurylsarcosine, and 0.72% 2-mercaptoethanol. A phenol extraction was conducted and following centrifugation, the aqueous phase was removed and diluted with isopropanol. Pelleted RNA was washed in cold 80% ethanol, dried and suspended in diethyl-pyrocarbonate-treated water. Denatured RNA samples were run in a denaturing 1.3% formaldehyde-agarose gels. Equal loading was assessed by ethidium bromide staining. The samples were transferred to a nylon membrane (Bio-Rad), cross-linked by Ultraviolet light and hybridized for 24 hours at 55°C with pre-hybridization solution (1% BSA [w/v], 35% 1M NaPO₄ [v/v], 7% SDS [w/v] and 30% de-ionized formamide). This was followed by hybridization (same temperature) with ³²P-ATP- labeled oligonucleotide probes for α-SMA and β-actin mRNA as described below.

MacMolly Tetra program (Soft Gene) was employed to create two 32-mer oligonucleotides (5'-TCCACAGGACATTCACAGTTGTGTGCTAGAGA-3' and 5'-CCATGCCAATCTCATTTTTCTTCTGGAAG-3') complementary to a sequence of α-SMA and β-actin mRNA 3' region respectively. The oligonucleotides were synthesized by Biotechnology service center (Hospital for Sick Children, Toronto). Probes were end-labeled with γ-³²P-ATP (3000 Ci/mmol; Dupont NEN) using 5' end labeling. For this purpose 2 µl of the probe (50 ng/µl) was added to 10 µl of d.H₂O, 2 µl of Polynucleotide kinase buffer (9700 units/ml), 6 µl of ³²P-ATP and finally 2 µl of polynucleotide kinase (FPLC pure, Pharmacia Biotech, Montreal, QU) and incubated for 30 minutes at 37°C. The cocktail was run through a nickcolumn (Sephadex G-50, Pharmacia Biotech.) to separate the labeled probe which was followed by counting the radioactivity. Finally, the probe was added to the blot in a hybridization bottle and hybridized overnight at 43°C. The blots were then washed twice with 0.5% SSC + 0.5% SDS in dH₂O, each time for 30
screens overnight. The blots were stripped and reprobed with $\alpha-^{32}$p-dCTP- labeled glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA for assessment of loading equality.

**Gel contraction Assay**

To determine the effect of TGF-β1 on remodelling of 3-dimensional anchored and floating gels, the contraction assay was conducted on triplicate cultures by adding $^{3}$H$_2$O to the culture media and by measuring radioactivity of the gels at certain time points. Samples of cell/collagen solutions (400 μl, 4×10$^5$ cells/ml) were pipetted into 35 mm tissue and non-tissue culture dishes. After polymerization gels were covered with α-MEM ± 15% FCS containing 1 μCi of $^{3}$H$_2$O (1 mCi/g, Dupont). Equilibration of radioactivity in the gels required 30 minutes. At the times indicated, the medium was removed, and the gels were rinsed quickly and dissolved in 0.5 ml of 1 M NaOH. The samples were neutralized with HCl, mixed with 2 ml scintillation solution and counted in a Beckman scintillation spectrometer. The initial gel volume was determined in gels without fibroblasts.

**Inhibition Assay**

To investigate the role of the $\alpha_2\beta_1$ integrin on TGF-β1-induced expression of $\alpha$-SMA, an inhibition assay was conducted using mouse monoclonal antibodies to block $\beta_1$ and/or $\alpha_2$ integrin subunits in the presence of TGF-β1. Gingival fibroblasts (7.5×10$^4$ cells/cm$^2$) were preincubated with mouse monoclonal antibodies for $\beta_1$ integrin (clone 4B4-RD1, 30 μl/100 μl α-MEM) and/or $\alpha_2$ integrin (clone P1H5, 10 μl/100 μl α-MEM) at room temperature and the cells were allowed to adhere to collagen-coated plastic surfaces of 35 mm Petri dishes. The next day media were removed, cultures were covered with α-MEM containing 10 ng/ml TGF-β1 supplemented with $\beta_1$ (1:30) and/or $\alpha_2$ (1:50).
the 3 day incubation with the same concentrations applied for preincubation. Each condition was prepared in triplicate. To analyse the antibody-induced changes of cell morphology, cells were observed with a phase contrast microscope (Nikon, Mississauga, ON) and photographs were taken on the 3rd day of incubation. At the end of treatment immunoblotting for α-SMA was conducted as described before.

**TGF-β Blockade**

To determine the specificity of the effect of TGF-β on α-SMA expression an anti-TGF-β neutralizing antibody was used to block the effect of TGF-β. Cells were plated on 35 mm collagen-coated dishes. After 24 hours media were replaced by α-MEM ± 10 ng/ml TGF-β, with or without anti TGF-β neutralizing antibody (15 µl/ml, R&D Systems). After 3 days α- SMA expression was quantified by western blot.

**ELISA**

To evaluate the levels of active and latent TGF-β, produced by fibroblasts and the effect of exogenous TGF-β, on these levels, a human TGF-β immunoassay (Quantikine, R&D Systems) was used which can detect a minimum of 5 pg of TGF-β/ml. Monolayer cultures of fibroblasts, 3-dimensional floating and anchored gels were prepared as described. Cells were incubated with α-MEM ± 15% serum with or without 10 ng/ml TGF-β, After 3 days cell culture supernatents were collected and triplicate samples were assayed with or without prior acid-activation of latent TGF-β. Acid activation was done by adding 0.1 ml 1 N HCL to 0.5 ml of cell supernatants for 10 minutes, followed by neutralization with 0.1 ml 1.2 N NaOH. TGF-β levels in these samples represents the total TGF-β activity (active + latent) whereas the direct assay of the conditioned media represents levels of active TGF-β.
and subtracted from activated samples of serum-containing conditioned medium. To determine the possible non-specific adherence of TGF-β₁ molecules to collagen, 10 ng/ml TGF-β₁ was incubated in collagen coated plates without cells and the medium was assayed after 3 days. To avoid attempting to measure higher levels of TGF-β₁ than the highest levels that could be accurately measured with the assay, the samples containing exogenous TGF-β₁ were diluted 1:5 and adjusted for the added TGF-β₁. Samples were analysed in a 96-well microtiter plate coated with recombinant human TGF-β₁ soluble receptor type II. The optical density of wells were determined with a microtiter plate reader set to 450 nm absorbance. The readings at 570 nm were subtracted from readings at 450 nm to correct for non-specific absorbance. The standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was determined by regression analysis.

Statistical Analysis

For quantitative data, means and standard errors of means were calculated. Comparisons between paired samples were performed using Student’s t-test (paired two sample for means).
To investigate the relationship between the development of intracellular tension and cell responsiveness to TGF-β₁ (compared to other isoforms, TGF-β₁ is the most abundant isoform in human platelets and is released by these cells into the wound area), 3 forms of collagen substrate with different resistance to cell generated forces were used: monolayer cultures, 3-Dimensional anchored gels and floating gels.

**α-SMA Content**

*Localization*

In a preliminary experiment, concentrations of 1, 5 and 10 ng/ml TGF-β₁ were tested to study the effect on the expression of α-SMA in monolayer cultures. Cells immunostained for α-SMA showed a dose-dependent response to TGF-β₁ and the optimal intensity of staining was observed with 10 ng/ml TGF-β₁. As this dosage has also been used in previous experiments to study TGF-β₁ regulation of α-SMA (Desmouliere et al., 1993; Orlandi et al., 1994) and is similar to levels found in healing wounds (Cromack et al., 1987), I decided to use this dosage for all subsequent experiments.

In monolayer cultures, compared to controls, TGF-β₁ (10 ng/ml for 3 days) promoted the development of stress fibers which were prominently stained for α-SMA. However the majority of α-SMA staining was diffusely distributed throughout the cell and thus was likely in monomeric form (Fig.1) as has been shown earlier (Arora and McCulloch, 1994). The TGF-β₁ enhancement of α-SMA staining was observed in both the presence and absence of 15% serum, however it was more obvious in +serum conditions. Confocal imaging of cells plated in anchored collagen gels and stained for α-SMA revealed essentially the same distribution as monolayer cultures. TGF-β₁ (10 ng/ml, 3 days) enhanced cell spreading into collagen gels which was accompanied by an increase in the intensity of α-SMA staining (Fig.2). Similar to monolayer cultures the
Figure 1. Cells on collagen coated plastic were incubated with α-MEM in the presence (A,B) or absence (C,D) of 15% serum, without (A,C) or with (B,D) 10 ng/ml TGF-β1 for 3 days. α-SMA was immunostained with anti-α-SMA antibody followed by a second conjugated FITC-goat anti-mouse antibody. Photomicrographs were obtained with a fluorescence microscope. (×400)
Figure 2. Confocal micrographs of the cells in anchored collagen gels were cultured without (A,C) or with (B,D; 10 ng/ml) TGF-β, in the presence (A,B) or absence (C,D) of 15% serum for 3 days. Subsequently, α-SMA was stained with anti-α-SMA antibody. Sections were obtained by optical sectioning at a nominal thickness of 1μm. (×650)
collagen gels that were stained for α-SMA did not show any significant change in cell morphology compared to controls, nor were there changes in the intensity of α-SMA staining in plus or minus serum conditions (Fig. 3).

It has been shown that reorganization of stress fibers is promoted by the development of intracellular tension (Gabbiani et al., 1971). Affinity labelling of filamentous actin by rhodamine-phalloidin in HGFs (monolayer cultures) treated with 10 ng/ml TGF-β1 for 3 days was measured by single cell photometry. There was a 2-3 fold increase in F-actin content compared to untreated controls (Fig. 4). Confocal imaging of cells in monolayer cultures, anchored or floating gels stained for filamentous actin, revealed the prominent development of stress fibers parallel to the long-axis of cells in monolayer cultures and to a lesser extent in anchored gels. Staining was significantly enhanced with TGF-β1, however this enhancement was not detectable in floating gels (Fig. 5), suggesting that the stress fibers reorganized and incorporated actin optimally when there was development of intracellular tension.
Figure 3. Cells in floating gels were incubated with α-MEM in plus serum (A,B) or minus serum (C,D) conditions without (A,C) or with (B,D; 10 ng/ml) TGF-β₁ for 3 days. Subsequent to α-SMA staining, confocal micrographs were taken by optical sectioning at a nominal thickness of 1μm. (×650)
Figure 4.

Average Intensity/Cell

<table>
<thead>
<tr>
<th>Condition</th>
<th>Average Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, +S</td>
<td>~40</td>
</tr>
<tr>
<td>b) TGF-β, +S</td>
<td>~60</td>
</tr>
<tr>
<td>c) Control, -S</td>
<td>~20</td>
</tr>
<tr>
<td>d) TGF-β, -S</td>
<td>~80</td>
</tr>
</tbody>
</table>

Figure 5.
or -serum (c,d) conditions without (a,c) or with (b,d; 10 ng/ml) TGF-β1 for 3 days. Filamentous actin was stained with TRITC-Phalloidin and intensity of staining was measured by quantitative spectrofluorimetry in 10 standard areas from three independent samples (mean ± s.e.m.).

* = p< 0.01  Significantly different from Control, +S
** = p< 0.01  Significantly different from Control, -S

Figure 5. Cells on collagen coated plates (A,B), anchored (C,D) and floating collagen gels (E,F) were exposed to α-MEM + 15% serum without (A,C,E) or with (B,D,F) 10 ng/ml TGF-β1 for 3 days. Filamentous actin was stained with TRITC-Phalloidin and micrographs were obtained with a fluorescence microscope. (x650)
To determine whether TGF-β1 induces quantitative changes of α-SMA content in HGFs, western blotting was performed and β-actin content was used for comparison and for normalization of protein loading. For cells grown on plastic without collagen coating the level of α-SMA was increased 2.5-fold by TGF-β1 treatment (10ng/ml, 3 days) in the presence of 15% serum (compared to control +S) and there was a 3-fold increase in -serum condition with TGF-β1 (compared to control –S; Fig. 6). HGFs grown on collagen-coated plastic also revealed a marked increase in the level of α-SMA after TGF-β1 treatment which was 9-fold higher in +serum condition and 25-fold higher in –serum cultures (Fig. 7). This enhancement of α-SMA content required 3 days. Indeed overnight incubation of HGFs on collagen-coated plastic with TGF-β1 (10 ng/ml) did not significantly increase α-SMA (Fig. 8). When cells were grown in 3-dimensional anchored collagen gels and treated with TGF-β1 (10 ng/ml), α-SMA protein level increased by 70% in +serum and 90% in –serum conditions (compared to control +S and control –S, respectively; Fig. 9). In fibroblasts plated in floating gels, TGF-β1 (10 ng/ml) did not induce a significant change in the level of α-SMA (± serum) (Fig. 10) which was consistent with the results of immunofluorescence staining.
Figure 6. Cells grown on tissue culture plastic were incubated with α-MEM in the presence (a,b) or absence (c,d) of serum, without (a,c) or with (b,d; 10 ng/ml) TGF-β1 for 3 days. Three independent samples (mean ± s.e.m.) were assayed for α-SMA content using western blot technique. β-actin content was used for normalization of protein loading.

* = p< 0.01  Significantly different from Control, +S
** = p< 0.01  Significantly different from Control, -S
Figure 7. Cells on collagen-coated plastic were incubated with α-MEM in plus serum (a,b) or minus serum (c,d) conditions without (a,c) or with (b,d; 10 ng/ml) TGF-β, for 3 days and analyzed for α-SMA protein level using western blotting. β-actin content was used for normalization of protein loading, (n=3 for each condition).

* = p< 0.01  Significantly different from Control, +S

** = p< 0.01  Significantly different from Control, -S
Figure 8. Cells on collagen-coated plates were incubated overnight with α-MEM alone (Control) or supplemented with 10 ng/ml TGF-β. Three independent samples (mean ± s.e.m.) were analyzed for α-SMA expression using immunoblotting and values were normalized to β-actin.
Figure 9. Cells in 3-Dimensional anchored collagen gels were incubated with α-MEM in presence (a,b) or absence (c,d) of serum, without (a,c) or with (b,d; 10 ng/ml) TGF-β, for 3 days. Western blotting analysis was used for assessment of α-SMA expression in three independent samples (mean ± s.e.m.) and values were normalized to β-actin.

* = p < 0.05  Significantly different from Control, +S

** = p < 0.05  Significantly different from Control, -S
Figure 10. Cells in floating collagen gels were incubated with α-MEM in presence (a,b) or absence (c,d) of serum, without (a,c) or with (b,d; 10 ng/ml) TGF-β1 for 3 days. Three independent samples (mean ± s.e.m.) were analyzed for α-SMA protein level using western blotting and values were normalized to β-actin.
The relative amount of \( \alpha \)-SMA per cell in populations of detached cells (by 0.01% trypsin) was estimated using flow cytometry. Negative controls were used to determine the signal above background thresholds (Fig. 11). In contrast to western blotting and immunofluorescence staining there was no significant difference between control and TGF-\( \beta_1 \) treated cultures in terms of either the mean fluorescence intensity of antibody-stained \( \alpha \)-SMA or for the percentage of \( \alpha \)-SMA +ve cells. Since the cells were detached from the substrate surface for flow cytometric analysis, I reasoned that cell detachment and reduction of intracellular tension may have reversed the TGF-\( \beta_1 \) induced up-regulation of \( \alpha \)-SMA expression.

To further investigate this observation, western blotting was performed on cell suspensions prepared by trypsinization. The time period from cell detachment to protein extraction was equal to the time that cells had been in suspended form before fixation for flow. Subsequent probing of the protein extracts for \( \alpha \)-SMA revealed no significant difference between controls and TGF-\( \beta_1 \) treated cultures, confirming the observation made by flow cytometry (Fig. 12). Based on evidence presented by Lin et al. (1997) I hypothesized that cell detachment induced by trypsin may have caused leakage of \( \alpha \)-SMA through transient membrane passages.

Consequently, cell suspensions were prepared using a non-proteolytic agent (EDTA, 0.5 mM) to detach cells. After separating cell pellets from the supernatants, western blotting for \( \alpha \)-SMA showed that the amount of \( \alpha \)-SMA in the cell pellets was increased ~2.5 fold by TGF-\( \beta_1 \) as seen earlier with the freshly prepared cell lysates. I also found that there are significant levels of immunoreactive \( \alpha \)-SMA in the supernatants which was increased by TGF-\( \beta_1 \) (Fig. 13). In contrast, \( \beta \)-actin was barely detectable in the supernatants.
Figure 11. Cells grown on collagen-coated plastic were incubated with α-MEM alone (Control) or supplemented with 10 ng/ml TGF-β₁ for 3 days. Single cell suspensions were stained with either no antibody (a) for autofluorescence, only FITC-conjugated second antibody (b) or primary anti-α-SMA antibody and FITC-conjugated second antibody (c,d). Mean fluorescence intensity and percentage of α-SMA positive cells were assayed using flow cytometry.
Figure 12. Cells on collagen-coated plastic were incubated with α-MEM alone (a) or with 10 ng/ml TGF-β₁ for 3 days (b). Subsequently, cells were trypsinized (0.01%) and re-suspended in α-MEM for 1 hour. Western blot analysis of cell suspensions was performed for α-SMA in three independent samples (mean ± s.e.m.) and values were normalized to β-actin.
Figure 13. Cells on collagen-coated plastic were exposed to α-MEM alone (a,c) or supplemented with 10 ng/ml TGF-β₁ for 3 days (b,d). Subsequently, the cells were detached from the plates using EDTA (0.5 mM) and resuspended in α-MEM for 1 hour. After separating the cell pellets from the supernatants, western blotting was performed for α-SMA and β-actin. Due to undetectable levels of β-actin in supernatants (control) density of α-SMA was not normalized to β-actin. (n= 3 for each condition)

* = p< 0.01  Significantly different from Control (supernatant)

** = p< 0.01  Significantly different from Control (pellet)
To assess whether TGF-β\textsubscript{1} induced α-SMA at the mRNA level, total RNA preparation from HGFs on monolayer cultures was separated by electrophoresis, blotted and hybridized with α- or β-actin specific oligonucleotide probes. For assessment of equal loading the blots were also sequentially hybridized with a labeled cDNA probe for GAPDH (Fig. 14). In cells treated with TGF-β\textsubscript{1} (10 ng/ml, 3 days), α-SMA bands were markedly increased compared to control cells and this increase was particularly noticeable in serum conditions. In contrast, β-actin mRNA levels were unchanged by TGF-β\textsubscript{1} treatments.
Figure 14. HGFs grown on collagen-coated plastic and incubated with α-MEM in +serum (a,b) or −serum (c,d) conditions without (a,c) or with (b,d; 10 ng/ml) TGF-β1 for 3 days were assayed for α-SMA at mRNA level. Northern blots were hybridized with the oligonucleotides specific for either human α-SMA or β-actin mRNA. For assessment of equal loading the blots were also hybridized with a labelled cDNA probed for GAPDH.
Blockade

Blocking of TGF-β with an anti-TGF-β neutralizing antibody that reacted with all TGF-β isoforms was conducted to determine whether TGF-β was the principal effector of α-SMA up-regulation. A 3 day incubation of cells with α-MEM + Antibody showed a small decrease in α-SMA content compared to controls (α-MEM alone). Incubation of TGF-β1 treated HGFs (monolayer cultures) with the antibody revealed a large and significant reduction in α-SMA levels compared to controls (Fig. 15). The results not only demonstrated the specificity of TGF-β in up-regulation of α-SMA expression but also suggested a possible role for endogenous TGF-β in the induction of α-SMA expression.
Figure 15. Cells grown on collagen-coated plastic were incubated with α-MEM (a,b) or α-MEM plus TGF-β1 (c,d; 10 ng/ml) without (a,c) or with (b,d; 15 μg/ml) anti-TGF-β1 neutralizing antibody for 3 days. Three independent samples (mean ± s.e.m.) were analysed for the expression of α-SMA using western blotting and values were normalized to β-actin.

* = p< 0.05  Significantly different from Control

** = p< 0.01  Significantly different from TGF-β1
For assessing the actual concentrations of active and latent forms of TGF-β₁ to which the cells were exposed under different culture conditions, ELISAs were performed on 3 independent activated and non-activated samples and the mean ± s.e.m. was computed for each condition (Table 2). Addition of TGF-β₁ (10 ng/ml) stimulated cells in all 3 types of collagen substrates to synthesize TGF-β₁, a large proportion of which was in active form. Comparison of + and −serum conditions within each model showed that the effect of exogenous TGF-β₁ was significantly higher in the presence of serum. In +serum conditions the increase of endogenous TGF-β₁ (total and active) was largest in monolayer cultures (total: 5.5 ng/ml; active: 4.2 ng/ml) and smallest in floating gels (total: 2.7 ng/ml; active: 2.6 ng/ml) both in control and TGF-β₁ treated cultures. The concentrations for anchored gels were less than monolayer cultures (total: 4.9 ng/ml; active: 4.0 ng/ml) and the difference was statistically significant in controls (p< 0.05) but not in TGF-β₁ treated cultures (p> 0.2). In −serum conditions there were no significant differences in the total and active levels of endogenous TGF-β₁ between the 3 models.
Table 1. Cells on collagen coated plastic, anchored and floating gels were incubated with α-MEM alone or supplemented with 15% FCS, with or without 10 ng/ml TGF-β1 for 3 days. To determine the concentrations of total and active forms of TGF-β1 in cell supernatants, ELISA was performed on activated and non-activated samples respectively. The amount of exogenous TGF-β1 was subtracted from the values obtained for TGF-β1 treated cultures. The table illustrates the levels of endogenous TGF-β1 which are expressed in pg/ml. (n=3 for each condition)

<table>
<thead>
<tr>
<th>Monolayer Cultures</th>
<th>+ Serum</th>
<th>- Serum</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TGF-β</td>
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<tr>
<td>Total</td>
<td>1060 ± 26.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5473 ± 44.4&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<tr>
<td>Active</td>
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<td>4236 ± 120&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<table>
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<th>Anchored Gels</th>
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<th>- Serum</th>
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<tr>
<td></td>
<td>Control</td>
<td>TGF-β</td>
</tr>
<tr>
<td>Total</td>
<td>728 ± 37.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4877 ± 189&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<tr>
<td>Active</td>
<td>&lt;5</td>
<td>3964 ± 140&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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</table>

<table>
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<th>Floating Gels</th>
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<th>- Serum</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TGF-β</td>
</tr>
<tr>
<td>Total</td>
<td>171 ± 17.5</td>
<td>2714.3 ± 83.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Active</td>
<td>&lt;5</td>
<td>2557.3 ± 55.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> = p<0.01 Significantly different from Control  
<sup>b</sup> = p<0.05 Significantly different from TGF-β1, -serum  
<sup>c</sup> = p<0.05 Significantly different from floating gels (TGF-β1 treated)  
<sup>d</sup> = p<0.01 Significantly different from floating gels (Control)
Expression

The $\alpha_2\beta_1$ integrin is a major collagen adhesion molecule in human gingival fibroblasts and is the most important integrin in cell adhesion and phagocytosis (Lee et al., 1996; Chou et al., 1996). As Heino et al. (1989) showed up-regulation of the $\beta_1$ family of integrins in some but not all human fibroblast lines, I determined the effect of TGF-$\beta_1$ on the total content and surface expression of $\alpha_2\beta_1$ integrin in HGFs. Flow cytometry analysis of cells grown on collagen-coated plastic for $\alpha_2$ and $\beta_1$ integrin subunits showed no significant differences of the mean intensity of staining or the % of +ve cells between controls and TGF-$\beta_1$ treated cells in either unfixed or fixed samples (Figs. 16,17). This result was also confirmed by immunoblotting of cells (monolayer cultures on collagen) for both subunits (Fig. 18). A possible explanation for these results could be that the collagen coating on the plates induced maximal expression of $\alpha_2\beta_1$ integrin; therefore further increases induced by TGF-$\beta_1$ would not be detected. Consequently, cells grown on tissue culture plastic and treated with TGF-$\beta_1$ were probed for $\alpha_2$ and $\beta_1$ subunits. The results revealed a ~2 fold increase in the expression level of both subunits after TGF-$\beta$ treatment compared to controls (Fig. 19).
Figure 16. Cells grown on collagen-coated plates were incubated with α-MEM alone (control) or supplemented with 10 ng/ml TGF-β₁ for 3 days. Single cell suspensions were stained with either no antibody (a) for autofluorescence, only FITC-conjugated second antibody (b) or primary anti-α₂-integrin and FITC-conjugated second antibody (c,d). Mean fluorescence staining and percentage of α₂-integrin positive cells were assayed in fixed (A) or unfixed (B) cells by flow cytometry to determine the total content or the surface expression of α₂-integrin subunit respectively.
Figure 17. Cells grown on collagen-coated plates were incubated with α-MEM alone (control) or supplemented with 10 ng/ml TGF-β₁ for 3 days. Single cell suspensions were stained with either no antibody (a) for autofluorescence, only FITC-conjugated second antibody (b) or primary anti-β₁-integrin and FITC-conjugated second antibody (c,d). Mean fluorescence staining and percentage of β₁-integrin positive cells were assayed in fixed cells using flow cytometry.
Figure 18. Cells grown on collagen-coated plastic were incubated with α-MEM alone (a,c) or supplemented with 10 ng/ml TGF-β1 (b,d) for 24 hours. Three independent samples (mean ± s.e.m.) were analyzed for α2-integrin (a,b) or β1-integrin (c,d) protein levels using western blotting and β-actin was used for normalization of protein loading.
Figure 19. Cells grown on tissue culture plastic were incubated with α-MEM alone (a,c) or supplemented with 10 ng/ml TGF-β (b,d) for 24 hours. Three independent samples (mean ± s.e.m.) were analyzed for α₂-integrin (a,b) or β₁-integrin (c,d) protein levels using western blotting and β-actin was used for normalization of protein loading.

* = p<0.01  Significantly different from Control (a)
** = p<0.01  Significantly different from Control (c)
Schiro et al. (1991) demonstrated a major role for $\alpha_2\beta_1$ integrin in reorganization of a hydrated collagen matrix. Therefore, I hypothesized that the $\alpha_2\beta_1$ integrin is involved in the up-regulation of $\alpha$-SMA induced by TGF-$\beta_1$. To test this hypothesis, cells were preincubated with the monoclonal antibodies P1H5 (which blocks the $\alpha_2$ subunit [Wayner et al., 1988]) and/or 4B4 (which blocks the $\beta_1$ subunit [Shimizu et al., 1990]) and treated with TGF-$\beta_1$ (10 ng/ml) for 3 days. Western blot analysis of $\alpha$-SMA levels normalized to $\beta$-actin contents demonstrated that preincubation with P1H5 reduced the level of $\alpha$-SMA induced by TGF-$\beta_1$ by 20% (compared to TGF-$\beta_1$ alone). However, treatment with 4B4 alone decreased TGF-$\beta_1$ induced expression of $\alpha$-SMA by 65% (compared to TGF-$\beta_1$ without antibody). Preincubation with both antibodies reduced the effect of TGF-$\beta_1$ to the level of controls (Fig. 20). The decrease in the expression of $\alpha$-SMA in the presence of 4B4 alone or combined with P1H5 was accompanied by a change in the cell morphology. Cells changed from well spread to rounded shapes and this alteration was most prominent toward the end of TGF-$\beta_1$ incubations when the maximum effect on $\alpha$-SMA expression was also observed (Fig. 21). These results were not caused by cell death as the viability of cells preincubated with antibodies and tested at the end of the incubations exceeded 95% as measured by trypan blue exclusion.
Figure 20. HGFs in suspensions were preincubated with either no antibody (a,b), only anti-α2-integrin antibody (3:10; c), only anti-β1-integrin antibody (1:10; d) or both antibodies (e) for 30 minutes and plated on collagen coated plastic in the presence of α-MEM (a) or supplemented with 10 ng/ml TGF-β1 (b,c,d,e) for 3 days. Antibodies were replenished once during the incubation period. Three independent samples (mean ± s.e.m.) were assayed for the expression of α-SMA.

* = p<0.01 Significantly different from Control
Figure 21. Photomicrographs of HGFs preincubated with $\alpha_2$ and/or $\beta_1$-integrin antibody. Control (A), TGF-$\beta_1$ alone (B), TGF-$\beta_1$ with either anti-$\alpha_2$-integrin antibody (C), anti-$\beta_1$-integrin antibody (D) or both antibodies (E). Micrographs were obtained on day 3 of treatment. (x150)
To determine whether there was a functional relationship between TGF-β₁-induced up-regulation of α-SMA and the ability of the cells to contract collagen matrices, a gel contraction assay was used. Contraction was determined by measuring gel volume based on the partitioning of $^{3}$H₂O between the gel phase and the surrounding medium following contraction (Nakagawa et al., 1989a). The rates of fibroblast contraction of anchored and floating gels (± 10 ng/ml, ± 15% serum) were compared in time course studies. In anchored gels and in the absence of serum, TGF-β₁ significantly increased the rate of contraction up to day 5 (88% compared to 60% in controls on day 3; Fig. 22a), although in both groups (± TGF-β₁), the gels exhibited maximum contraction by 8 days. Serum (15%) markedly enhanced contraction of anchored gels compared to -serum conditions (26% in -serum vs. 58% in +serum condition after 6 hours; Fig. 22b). Serum also abrogated the effect of TGF-β₁ that was observed on day 3 in the absence of serum. In serum-containing anchored gels, contraction showed a biphasic pattern in which there was a rapid contraction within 24 hours after plating the gels (90% contraction) which was followed by a slow contraction that was complete by 5 days.

Floating gels demonstrated a very fast initial contraction (Fig. 23) which was enhanced with serum (60% in -serum vs. 83% in +serum condition after 6 hours). The contraction of floating gels followed the biphasic pattern described for anchored gels and TGF-β₁ significantly increased the rate of contraction between days 3 to 5 in minus serum conditions (>98% compared to 76% in controls on day 3). However there was no effect of TGF-β₁ in plus serum conditions.

A comparison between anchored and floating gels showed that the rate of contraction was slower in anchored gels (33% in anchored vs. 60% in floating gels after 6 hours) without serum, however in +serum conditions there was no obvious difference between the two types of gels.
Figure 22. HGFs in anchored collagen gels were incubated in α-MEM containing $^3$H$_2$O (1 μCi/ml) with or without TGF-β (10 ng/ml) in the absence (a) or presence (b) of serum (15% serum). At the times indicated gel volume was determined using scintillation counting of labelled gel. For each time point n = 3

* = p< 0.05   Significantly different from Control (days 3 and 5)
Figure 23. HGFs in floating collagen gels were incubated in α-MEM containing $^3$H$_2$O (1 μCi/ml) with or without TGF-β (10 ng/ml) in the absence (a) or presence (b) of serum (15% serum). At the times indicated gel volume was determined using scintillation counting of labelled gel. For each time point n = 3

* = p< 0.05 Significantl differnet from Control (days 3 and 5)
My central finding is that the ability of TGF-β₁ to increase α-SMA expression in fibroblasts is determined by the physical resistance of the substrate to cell-generated forces. α-SMA is an abundant actin isoform in vascular smooth muscle cells (Rubenstein et al., 1990) and is the most common smooth muscle cell marker expressed by myofibroblastic populations (Desmouliere et al., 1994). The presence of α-SMA expressing myofibroblasts in a tissue such as granulation tissue is accompanied by rapid matrix remodeling and is also characterized by the development of tension (Sappino et al., 1990). Indeed in granulation tissue, increased tension leads to wound contraction, a critical process for mammalian survival. While the modulatory role of TGF-β on myofibroblast differentiation and expression of α-SMA has been studied previously in monolayer cultures (Bjorkerud, 1991; Desmouliere et al., 1993; Orlandi et al., 1994), there are no reports on the study of cells responding to TGF-β under different levels of tension. Therefore it was of interest to determine the role of tension in TGF-β-induced α-SMA expression. In this study, I used three different tissue culture systems with variations in substrate deformability to provide study models in which cells would be exposed to different levels of mechanical stress. These models included monolayer cultures of fibroblasts on collagen-coated plastic (high tension), 3-dimensional anchored (moderate tension) and floating (low tension) collagen gels.

Model systems

Fibroblasts grown in collagen gels develop a 3-dimensional network in the gel and unlike fibroblasts in monolayer cultures exhibit morphological features similar to those of fibroblasts in vivo (Bell et al., 1979; Bellows et al., 1981; Tomasek et al., 1982). Moreover, collagen gels can be reorganized by fibroblasts which provides a useful model for studies of
studies of wound healing. These two models (anchored and floating collagen gels) demonstrate distinct mechanical features due to differences in their deformability. Based on morphological and biosynthetic data presented by Nakagawa et al. (1989), anchored gels resemble granulation tissue whereas floating gels resemble resting dermis. In this study I analyzed the effect of TGF-β1 on cell morphology and expression of α-SMA in the 3 models described above. Collagen type I was used as the substrate in all 3 models, thereby validating comparisons despite differences in the mechanical properties of the gels.

The models demonstrated different levels of resistance to the tractional forces exerted by fibroblasts. Collagen-coated plastic is a rigid substrate in which there is no observable alteration of the dimensions of the gel and in which there is high resistance against deformation. In collagen gels, based on the results of $^{3}$H$_2$O exclusion, anchored gels showed a 60% reduction in volume after 3 days. This was significantly less than the relative reduction of volume in floating gels which was >90% during the same time period. Thus deformability was highest in floating gels and was lowest in monolayer cultures. I assume on the basis of these data that the deformability of anchored gels lies somewhere in between floating gels and monolayer cultures.

Different substrate deformability translates into different levels of intracellular tension. The development of tension inside the cell can be characterized by the reorganization of stress fibers as the force-generating elements (Burridge, 1981). Fibroblasts grown on rigid substrates acquire characteristics of myofibroblasts and develop prominent stress fibers (Schmitt-Graff et al., 1994). Also in vivo, it has been shown that cells respond to tension with the appearance of stress fibers (Gabbiani et al., 1971; 1975). Further, in this study, confocal imaging of cells stained for filamentous actin revealed the development of prominent stress fibers in monolayer cultures, far less development in anchored gels and
relationship between the degree of substrate deformability and the development of intracellular tension.

**Effect of tension and TGF-β₁ on α-SMA expression and localization**

The principal finding from micrographs of cells stained for α-SMA or for stress fibers was the tension-dependent enhancement of cell spreading and development of stress fibers in response to TGF-β₁. Further, stress fibers were prominently stained for α-SMA, although the majority of α-SMA was more diffusely distributed and was not restricted to fibrillar elements. These findings suggest that α-SMA was largely present as monomer as has been shown earlier (Arora and McCulloch 1994). The results of immunoblotting for α-SMA were consistent with immunofluorescence staining and showed the maximal regulatory effect of TGF-β₁ on α-SMA expression in monolayer cultures, an intermediate effect in anchored gels and no detectable effect in floating gels. Therefore, it appears likely that TGF-β₁ stimulates the transition of fibroblasts to a more myofibroblastic appearance when cells are exposed to tension, but not when they are in unstressed, resting conditions.

In these experiments cells were exposed to TGF-β₁ for 3 days, however incubations of cells for 24 hour with TGF-β₁ did not induce any detectable change in the protein level of α-SMA in monolayer cultures. This observation raised the question of how direct was the effect of TGF-β₁ on α-SMA expression. If 24 hours incubation of cells with TGF-β₁ was not long enough to increase α-SMA levels, then it might act indirectly, perhaps by inducing the production of other growth factors such as PDGF. Although TGF-β can regulate the synthesis of PDGF in some cell types (e.g. endothelial cells; Roberts and Sporn, 1989), PDGF is unable to induce α-SMA expression in dermal fibroblasts in vitro (Sappino et al., 1990).
blot analyses were performed on monolayer cultures. Compared to β-actin mRNA levels which were unaltered, the density of the bands for α-SMA were markedly increased after treatment with TGF-β1 for 72 hours compared to controls. Overall, the regulatory effect of TGF-β1 on α-SMA content both at the protein and mRNA levels were more prominent in minus serum conditions. This effect could be due in part to the presence of several factors in serum such as α2-macroglobulin (O'Conner and Wakefield, 1987) that can bind to active TGF-β and inactivate it.

To determine whether the effect of TGF-β observed in these experiments was specific, a neutralizing antibody was used to block its action. Compared to controls, there was a significant reduction in α-SMA content when TGF-β1 treated cells were incubated with the antibody. Further, control cells (no TGF-β1 treatment) showed a reduction of α-SMA content. These observations indicate not only the specificity of TGF-β in mediating an increase of α-SMA content but also a possible role for endogenous TGF-β. Therefore it was of considerable interest to determine the actual concentrations of TGF-β1 to which the cells were exposed in the different models. Previous studies have shown that TGF-β is present in the extracellular matrix both in active and latent forms but only the active form is able to bind to TGF-β receptors (Wakefield et al., 1987). As the latent form can be activated in vitro by acidification (Lawrence et al., 1985), I quantified both active and latent forms of TGF-β1 in culture medium using an ELISA. The data indicated that exogenous TGF-β1 stimulates the cells to produce TGF-β1, the majority of which is in an active form. These results are consistent with the findings of Lin et al. (1995) showing that TGF-β1 amplifies its own production. This stimulatory effect of exogenous TGF-β1 was prominently enhanced by serum. Moreover, in the presence of serum, the type of culture system significantly altered the level of endogenous TGF-β1: the highest levels of active and latent
TGF-β1 were observed in monolayer cultures and the lowest levels were in floating gels. The concentrations for anchored gels was less than monolayer cultures but the difference was not statistically significant. Further, there were high levels of active TGF-β1 in floating gels (~2 ng/ml), indicating that the lack of the effect of TGF-β1 on the expression of α-SMA observed in floating gels was not simply due to inadequate levels of active TGF-β1 in the culture. However it is possible that in floating gels there might be a decrease in the level of surface receptors or their ability to become activated in response to TGF-β1, as has been shown for PDGF receptors in collagen gels (Lin and Grinnell, 1993).

To determine if there were subpopulations of cells with varying levels of α-SMA in response to TGF-β1, flow cytometric analyses were conducted on trypsinized single cell suspensions prepared from monolayer cultures. Surprisingly, there was no significant difference between controls and TGF-β1 treated-cultures in terms of either mean fluorescence intensity or the percentage of α-SMA positive cells. This observation was confirmed by immunoblotting cell suspensions prepared with identical methods as the cells used for flow cytometry. As I had demonstrated a relationship between tension and TGF-β1-induced regulation of α-SMA, I considered the possibility that these unexpected results were due to the release of intracellular tension after cell detachment. Thus trypsinization and cell rounding might have reversed the stimulatory effect of TGF-β1 on synthesis of α-SMA. However, due to the relatively short time period from cell detachment to α-SMA analysis in these experiments (<2 hours), this explanation seemed unlikely.

Another possible explanation was suggested by the studies of Lin and Grinnell (1997) who showed that in fibroblasts cultured in contracting collagen gels, mechanical perturbations induced the formation of transient membrane passages permeable to small molecular mass compounds. They suggested that the rapid reorganization of cell-matrix adhesion sites in stress-released gels may be involved in the formation of these passages. As
fibers and intracellular tension, it appears possible that the flow cytometric data were due to leakage of α-SMA monomer through membrane passages after cell detachment. My analyses of α-SMA content in cell supernatants after detaching the cells by EDTA showed high levels of α-SMA which were significantly higher in TGF-β₁ treated cultures. In contrast, β-actin levels in cell supernatants were very low both in controls and TGF-β₁-treated samples. There are several lines of evidence that could explain why α-SMA but not β-actin leaks through the membrane openings. First, α-SMA is found near the surface of endothelial cells in culture (Moroianu et al., 1993) and in focal adhesion contacts in fibroblasts (Rommov-Jessen and Peterson, 1996) and thus is likely to be located close to membrane passages. Second, immunofluorescence staining showed a diffuse distribution of α-SMA throughout the cell and previous observations have demonstrated that the majority of α-SMA in gingival fibroblasts is in a monomeric form (Arora and McCulloch 1994); in contrast, β-actin is found mainly in filaments. Thus compared to β-actin, I suggest that α-SMA is much more likely to exit the cells through the membrane passages.

Based on the experiments described above it is possible that the different α-SMA responses to TGF-β₁ which were observed between monolayer cultures and anchored gels were due in part to the varied methods of protein extraction. In monolayer cultures the cells were lysed and scraped directly from the surface of the plate so the results of immunoblotting showed the whole α-SMA content of attached cells. However the method of protein extraction in anchored gels (enzyme treatments) might have promoted the leakage of α-SMA through transient cell membrane openings during the process of releasing the cells from the gels and subsequent harvesting. Although this possibility exists, I note that in floating gels and anchored gels the protein extraction methods were identical but that there were still marked differences in α-SMA content. Consequently, I believe that
Gel Contraction

The gel contraction assay provided a functional model to relate the role of TGF-β₁-induced expression of α-SMA and the ability of the cells to reorganize their substrate. The rationale for focusing on α-SMA and gel contraction is based in part on the dependence of this phenomenon on expression of the α-SMA actin isoform (Arora and McCulloch 1994). Since gel contraction cannot be measured in monolayer cultures, I restricted my analyses to anchored and floating collagen gels. In addition to α-SMA expression, the cell-generated force required for collagen gel contraction is also dependent on several factors including cell type (Bellows et al., 1981), cell number (Bell et al., 1979), passage number (Murphy and Daniel, 1987), serum (Steinberg et al., 1980) and different growth factors. TGF-β (Montesano and Orci, 1988) and PDGF (Gullberg et al., 1990) can stimulate collagen gel contraction while FGF (Finesmith et al., 1990) and γ-interferon (Gillery et al., 1992) inhibit this process. As serum contains several of these factors that can influence collagen gel contraction, both plus and minus serum conditions were used to analyze the role of TGF-β₁ in gel contraction. Comparison of contraction rates showed that in minus serum conditions, anchored gels contracted more slowly than floating gels which is consistent with the findings of Nakagawa et al. (1989) and reflects the difference in the deformability of the substrate. However the addition of serum abrogated TGF-β₁-induced differences in contraction rates for both types of collagen gel models, presumably because of the presence of multiple factors in serum that can influence gel contraction such as PDGF and lysophosphatidic acid.

In floating gels, TGF-β₁ significantly increased the rate of contraction in minus serum conditions between days 3 to 5 (>98% compared to 76% in controls on day 3) which
was consistent with the finding of Tingstrom et al. (1992). However there was no difference between controls and TGF-β1 treated gels in plus serum conditions. This result was in contrast to the finding of Montesano et al. (1989) who reported stimulation of gel contraction by TGF-β1 in the presence of serum. The difference between my data and the previous studies could be due to difference in cell types, passage number (Murphy and Daniel, 1987), the type of measurement system (i.e. ³H₂O exclusion versus gel diameter) and the size of the collagen gels. Further, it is notable that TGF-β1 pretreated palatal fibroblasts manifest rapid contraction of collagen gels in the absence of TGF-β1 (Yokozeki et al. 1997), suggesting that this cytokine stimulates contraction of floating gels by converting normal fibroblasts into a more contractile phenotype. Consequently, the effect of previous cell selection in vitro cannot be ignored and it is conceivable that depending on individual batches of cells, there will be wide variations of clonal type and responsiveness to TGF-β1.

To my knowledge, there have been no previous reports on the effect of TGF-β1 on contraction of anchored gels. My data on the contraction of anchored gels showed that in minus serum conditions, TGF-β1 significantly increased the rate of contraction up to day 5 (88% compared to 66% in controls on day 3). However, the addition of serum markedly enhanced the rate of contraction and abrogated the effect of TGF-β1. This is most likely due to the presence of PDGF in serum which has been shown to induce a more rapid contraction of collagen gels than TGF-β1 (Tingstrom et al., 1992). Since there is no evidence of PDGF up-regulation of α-SMA in the literature, it is conceivable that the effect of PDGF on contraction of collagen gels is independent of α-SMA expression by fibroblasts.

**Integrins**

The β₁ family of integrins are the major adhesion molecules that mediate fibroblastic interactions with collagen (Wayner and Carter, 1987). These interactions play an important
contraction of FPCLs (Gullberg et al., 1990; Klein et al., 1991). Among the members of the $b_1$ family of integrins, $\alpha_2\beta_1$ is believed to play a pivotal role in reorganization of collagen matrices as it is selectively up-regulated on fibroblasts grown in 3-dimensional collagen gels (Klein et al., 1991). Further, cells expressing low levels of $\alpha_2\beta_1$ are unable to contract collagen gels (Schiro et al., 1991). Moreover, Heino et al. (1989) have shown that TGF-\(\beta_1\) upregulation of $b_1$ is cell-type specific. These data led me to question whether TGF-\(\beta_1\) up-regulates $\alpha_2\beta_1$ in gingival fibroblasts and if the increased expression of $\alpha_2\beta_1$ is involved in the $\alpha$-SMA modulation induced by TGF-\(\beta_1\).

Flow cytometric and western blot analyses of $\alpha_2\beta_1$ in fibroblasts grown on collagen-coated plastic showed no effect of TGF-\(\beta_1\) on both cell surface expression and the total content of $\alpha_2$ and $\beta_1$ subunits. The data from suspended cells and cells remaining attached to the substrate were similar, showing that cell detachment did not affect expression levels as it did for $\alpha$-SMA. Thus the increased $\alpha$-SMA levels induced by TGF-\(\beta_1\) is not because of increased integrin expression and the presence of more adhesive contacts into which $\alpha$-SMA-containing filaments could insert.

In view of the data of Heino et al. (1989) showing that certain fibroblast lines express higher levels of $\alpha_2\beta_1$ after TGF-\(\beta_1\) treatment, I considered whether gingival fibroblasts grown on collagen may already express maximal levels of $\alpha_2\beta_1$ and that therefore TGF-\(\beta_1\) could not further increase integrin levels. Consequently I analysed $\alpha_2\beta_1$ content in cells grown on tissue culture plastic as done by Heino et al. (1989). Western blotting demonstrated a 2-fold increase in protein levels for both subunits. Thus TGF-\(\beta_1\) can increase $\alpha_2\beta_1$ expression but this effect is dependent on the substrate.

In addition to possible changes in surface levels of integrins, some cytokines can alter the affinity of integrins for collagens (e.g. TNF-\(\alpha\); Chou et al. 1996). Indeed integrin
lymphocytes leads to activation but no change of surface levels of β₁ integrins (Shimizu et al., 1990). In contrast, integrins may become inactive but persist on the cell surface such as occurs for α₂β₁ integrins in teratocarcinoma cells (Dahl et al., 1989). These examples show that integrin activation and inactivation can be mediated from inside the cell. Therefore, it is conceivable that TGF-β₁ may regulate the activation of α₂β₁, presumably by accompanying changes in cell morphology and actin cytoskeletal rearrangements.

To assess whether α₂β₁ is involved in the process of TGF-β₁-induced regulation of α-SMA, α₂ and β₁ subunits were blocked with monoclonal antibodies. Western blot analyses revealed a large decrease in the level of α-SMA after β₁ antibody incubation and a smaller reduction with α₂ antibody compared to samples treated only with TGF-β₁. Based on previous studies, β₁ antibody is a more effective blocking antibody compared to α₂ antibody which may partly explain the marked reduction of α-SMA in the presence of β₁ but not α₂ antibody alone. Combined treatment with the two antibodies reduced α-SMA content to the level of controls. The reduced α-SMA content was accompanied by a change in cell morphology from well-spread to rounded. The altered cell morphology was more noticeable towards the end of the incubation with TGF-β₁ when the maximum effect on the expression of α-SMA was expected. These results from blockade of the α₂β₁ integrin further support the suggestion that the generation of intracellular tension is a requirement for the regulation of α-SMA by TGF-β₁. Inhibition of cell spreading and development of stress fibers through the use of blocking antibodies against α₂ and β₁ subunits significantly reduced the effect of TGF-β₁ on α-SMA expression.

Mechanocoupling and transcriptional control

Currently, it is unknown how variations in intracellular tension result in variations in the levels of α-SMA mRNA and protein in response to TGF-β₁. The TGF-β family of
receptors on the cell surface that transduce TGF-β binding into intracellular signals (Massague et al., 1994). TGF-β₁ can increase α-SMA expression in smooth muscle cells and this requires binding of an unknown factor to a TGF-β control element along with the binding of serum response factors to two CArG elements in the promoter region (Hautmann et al., 1997). Conceivably, a number of mechanical signalling systems serve to restrict either TGF-β₁ binding, signal transduction, transcription factor binding to the α-SMA promoter or transcription of α-SMA mRNA to only those cells that have developed significant intracellular tension. As the mechanical features of the ECM (such as its resistance to cell-generated tensile forces) can influence the biological activity of cells by altering their signal transduction machinery or modulating the cytoskeleton, I will speculate below on four possible mechanotransduction models that might be involved in the tension-dependent regulation of α-SMA induced by TGF-β₁.

1. Mechanosensitive ion channels have been demonstrated in different cell types and these include stretch-activated ion channels in skeletal muscle cells (Guhary and Sachs, 1985), vascular endothelial cells (Lansman et al., 1987) and fibroblasts (Stockbridge and French, 1988; Arora and McCulloch, 1994). Cell shape and the degree of cell spreading have been shown to influence the intracellular Ca²⁺ concentration in endothelial cells (Schwartz et al., 1993). Moreover, changes in cell shape and reorganization of cell-matrix adhesion sites in contracting fibroblasts induce the formation of membrane passages that might be involved in Ca²⁺ uptake (Lin et al., 1997). Alterations of intracellular Ca²⁺ concentration is an important signaling mechanism and may dramatically regulate a variety of downstream events. For example, the activity of some transcriptional factors such as the activator protein AP-1 depends on increased Ca²⁺ (Ghosh and Greenberg, 1995). Further, AP-1 binding is involved in the activation of the α-skeletal actin promoter in myocytes
Therefore tension-induced changes of intracellular ion levels may be involved in TGF-β₁-induced expression of α-SMA, possibly by regulating the activity of signaling molecules involved in this pathway.

2. Another possible mechanism may involve tension-induced changes in the arrangement of the cytoskeleton. A rigid substrate promotes the formation of stress fibers, a phenomenon that is due in part to the reorganization of adhesion molecules into focal adhesion complexes (Burridge, 1986). As stress fibers are connected to focal adhesions via actin binding proteins such as α-actinin, vinculin and talin, the rearrangement of focal adhesions may change the kinetics and thermodynamics of actin filament realignment leading to the formation of large actin bundles. It has been suggested that changes in cytoskeletal geometry can influence signal transduction pathways in a number of ways including the regulation of the cytoplasmic distribution of signaling molecules and therefore their ability to chemically interact and modulate transcription (Ingber, 1997). In this study, in addition to the differences in substrate deformability between the 3 collagen models, there is a major difference in cell to matrix interactions between the monolayer cultures and the 3-dimensional collagen gels. In monolayer cultures, the cells are interconnected with their substrate only through the basal surface; the lateral surfaces are involved in cell to cell interactions (i.e. gap junctions or adherence junctions). However in collagen gels, all cell surfaces are involved in cell to matrix and cell to cell interactions. Therefore, the difference in the distribution of matrix adhesion sites may also be partly responsible for the different levels of cellular responses to TGF-β₁ observed between monolayer cultures and collagen gels due to dramatic variations in cytoskeletal geometry.

3. An alternative explanation that is allied to the #2 proposal is based on a recent study by Bershadsky et al. (1995) who showed that autoregulatory cytoplasmic control of
acyn synthesis depends on the state of actin assembly. In another words, increased actin assembly leads to the up-regulation of actin synthesis. My results from affinity labeling of filamentous actin and immunofluorescence staining for α-SMA showed a tension-dependent reorganization of stress fibers and incorporation of α-SMA into the stress fibers in response to TGF-β1. Therefore, the incorporation of monomeric α-SMA into stress fibers in tense cells is much more likely to lead to up-regulation of α-SMA expression.

4. The mechanical connections between the cell surface, cytoskeleton and nucleus, when interpreted on the basis of the so-called tensegrity model, could explain how changes in cell morphology can directly influence nuclear shape (Ingber et al., 1993). Changes of nuclear structure are believed to alter gene expression in different ways. For example, it might act by altering nucleocytoplasmic transport through nuclear pores (Feldherr and Akin, 1993). Therefore, mechanical-induced changes of nuclear shape might regulate the expression of cytoskeletal genes involved in cell contraction.

Further studies are required to elucidate how changes in surface receptors and cellular architecture may affect gene regulation. My results from integrin inhibition assay have established the involvement of α2β1 integrin in TGF-β1-induced regulation of α-SMA and that it is not simply due to increased expression of the receptor. Thus it will be of interest to investigate TGF-β1 induction of α2β1 integrin activation and its role in α-SMA expression. Further, based on #3 mechanotransduction model described above, the state of α-SMA assembly may control the expression of α-SMA by a feedback control mechanism. Therefore it may be important to test this pathway by altering the thermodynamics of actin assembly and assessing its subsequent effects on α-SMA expression. Finally, to achieve a better understanding of TGF-β1 regulation of α-SMA expression more must be elucidated in regards to the transcriptional effect of TGF-β1 on α-SMA gene and the transcription factors associated with it.
situations (e.g. wound healing) myofibroblast differentiation is modulated locally by microenvironmental stimuli such as growth factors and mechanical stress. Therefore, it is conceivable that further studies along these lines may lead to programming efficient therapeutic strategies for enhancement of wound healing and prevention of pathologic scarring in the future.
CONCLUSION

1. TGF-β₁ enhances cell spreading, development of stress fibers and increase of α-SMA content in monolayer cultures and to a lesser extent in anchored collagen gels, but not in floating collagen gels.

2. Development of mechanical stress due to the physical resistance of the substrate to cell-generated force is required for TGF-β₁-induced up-regulation of α-SMA.

3. TGF-β₁ stimulates the contraction of anchored and floating collagen gels in the absence of serum, however the effect is more significant in anchored collagen gels.

4. The stimulatory effect of TGF-β₁ on its own production is significantly altered by the development of mechanical stress in the substrate.

5. α₂β₁ integrin is required for TGF-β₁ induction of α-SMA presumably by promoting cell spreading and development of intracellular tension.
V: REFERENCES


