Chronic *in vivo* Overexpression of TGF-β_1 Using a Retroviral Expression Vector

by
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CHRONIC *in vivo* OVEREXPRESSION OF TGF-B_1 USING A RETROVIRAL EXPRESSION VECTOR

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Abstract

There are numerous causes of progressive pulmonary fibrosis but the most common form is called idiopathic pulmonary fibrosis (IPF). The etiology of IPF is unknown and it is relentless and lethal. The proposed pathogenesis of IPF is that following tissue injury there is recruitment of inflammatory cells that are activated to release proinflammatory and fibrogenic cytokines. Of these cytokines TGF-β_1 is not only a mitogen for immature fibroblasts but also a chemoattractant for fibroblasts and induces connective tissue synthesis which leads to fibrosis.

In an early pulmonary injury response TGF-β_1 is aberrantly present in alveolar macrophages. However, in progressive pulmonary fibrosis where there are advanced lesions, TGF-β_1 is present in alveolar macrophages and epithelial cells irrespective of the etiology. Although we have demonstrated that increased expression of TGF-β_1 by alveolar macrophages is important for the pathogenesis of pulmonary inflammation and fibrosis, we sought to determine if aberrant expression of TGF-β_1 by alveolar epithelial cells was also important in regulating the inflammation and fibrosis. TGF-β_1 is usually secreted non-covalently associated with a latency associated peptide (LAP) that renders TGF-β_1 biologically inactive. For TGF-β_1 to be biologically effective the LAP has to be removed. To chronically overexpress TGF-β_1 we developed three retroviral vectors containing the cDNA for TGF-β_1 for *in vivo* administration and transfection. One vector contained the entire latent TGF-β_1 insert while a second contained an insert mutagenized at Cys 223 and Cys 225 of the LAP in TGF-β_1. This mutagenesis leads to an abnormal LAP and thus secretion of TGF-β_1 in an active form. A retroviral control vector that

contained no TGF-β_1 insert was also constructed. The viral particles were initially packaged using ψ_2 cells and their titers were calculated in colony forming units/ml (CFU/ml). Infectivity of the viruses was tested in fibroblast cell lines, NIH 3T3 and 10T1/2 as well as alveolar epithelial cell line A549 cells, grown in selective media. This resulted in 3 clones of NIH 3T3 , 3 clones of 10T1/2 fibroblasts and 3 clones of A549 cells. The TGF-β_1 released into the conditioned media by stably transfected NIH 3T3, 10T1/2 and A549 cells was assayed using an ELISA kit which quantitated the amount of active TGF-β_1 released by the cells. Our findings demonstrated that there was no detectable TGF-β_1 secreted from any of the clones. The secretion of TGF-β_1 requires glycosylation sites in the pre-pro region of the TGF-β_1 precursor. The lack of secretory TGF-β_1 may have been due to alteration of sites present in the pre-pro region of TGF-β resulting in intracellular accumulation of TGF-β_1 but not secretion.

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BCP	bovine calf plasma
bp	base pair
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CM	conditioned media
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
fmoles	fentomolar
FBS	fetal bovine calf serum
HeBS	hepes buffered solution
kb	kilo base
kDa	kilo dalton
LAP	latency associated peptide
LTBP	latent TGF-β binding protien
LTGF-β	latent transforming growth factor beta
M	molar; moles L^{-1}
ml	milliliter
mem	minimal essential media
mM	milli molar
ng	nanogram
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PEG	polyethylene glycol
pM	pico molar
TBE	tris/borate (buffer)
v	volts
μCi	microcurie
μL	microliter
μM	micromolar

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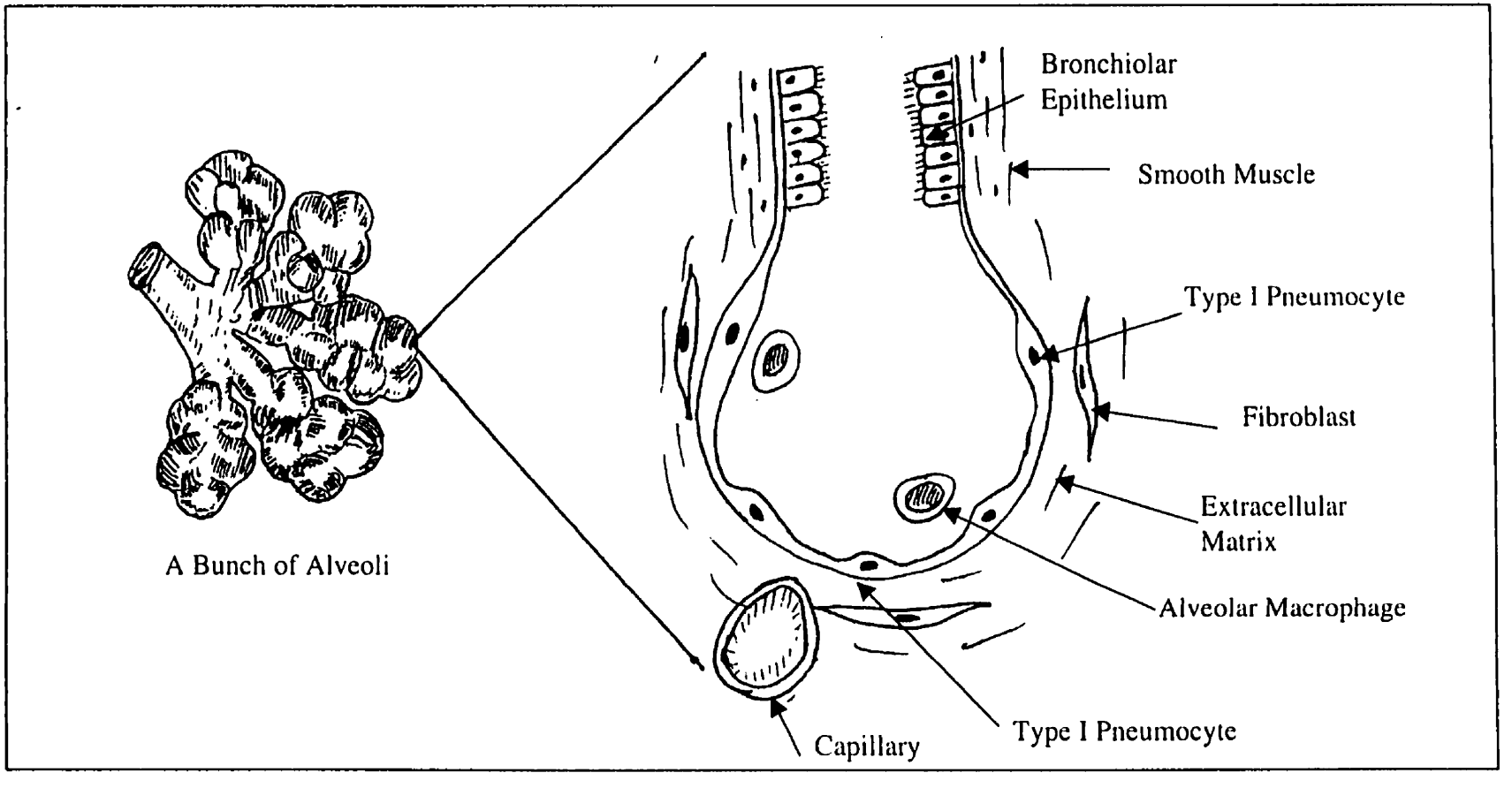
1. Introduction

1.1. Normal Lung Architecture

The most peripheral portion of the lung is composed of alveoli (Khalil and Greenberg, 1991). At the alveolus there is a transition from the columnar bronchiolar epithelium to the type I and type II alveolar epithelial cells that line the alveolar wall in a continuous single layer (Schneeberger, 1997). Within the alveolus, macrophages are the most prominent cell type while fibroblasts and extracellular matrix are present in the interstitial space (Figure 1). Type I pneumocytes cover 90% of the alveolar surface and are flat cells through which gas exchange predominately occurs (Schneeberger 1997). Type I pneumocytes can repair minor plasma membrane damage but are unable to regenerate after severe injury. Type II pneumocytes cover the remainder of the alveolar epithelial surface and are cubodial cells that produce surfactant, a surface active material that prevents alveolar collapse. Type II pneumocytes can replicate and increase in number after injury and destruction of type I cells. The epithelial cells rest on a basement membrane and are in close proximity to the pulmonary capillaries (Mason and Shannon, 1997). The close approximation of alveoli to the capillaries facilitate gas exchange (Simon and Paine, 1995). Under normal conditions, the airway epithelial lining is well differentiated and only a few cells undergo mitosis at the same time (Laitinen et al, 1994)

Although within the normal lung there are a number of inflammatory cells present more than 90% are alveolar macrophages. Alveolar macrophages are pluripotential cells that function as an important mechanism in the lower respiratory tract to remove inhaled organisms and particulates (Snider, 1983). Normal alveolar macrophages are quiescent

Figure 1: A Normal Bronchiole, Alveolus and Interstitium



Reference: Khalil and Greenberg, 1991

in healthy people and do not secrete significant levels of cytokines. However, once they are stimulated either *in vivo* or *in vitro*, they release a variety of mediators that may play a role in inflammatory and immune processes (Hunninghake et al, 1980). In addition to cytokines, activated macrophages release a variety of other mediators as well, including reactive oxygen, nitrogen intermediates, hydrolytic enzymes, and lipids (Nathan, 1987). These mediators act in some instances in concert with cytokines to promote tissue injury and in other instances tissue repair (Holian and Scheule, 1990; Laskin and Pendino, 1995; Nathan, 1987). In normal lung, lymphocytes make up the remaining 5 % of the effector cells which are primarily T lymphocytes and are not activated (Crystal et al, 1984). However, upon activation T lymphocytes also release a number of mediators. The role of activated lymphocytes in pulmonary fibrosis has not been described to be as important as activated alveolar macrophages.

The alveolar interstitum is a region made up of fibroblasts and connective tissue matrix that together represent almost 50 % of the tissue volume of the alveolar wall (Crapo et al, 1982). In normal lung, fibroblasts account for 37 % of all parenchymal cells and occupy approximately two thirds of the volume of the interstitium (Seyer et al, 1976). The most abundant interstitial matrix components are collagen types I and III which are structural macromolecules normally present in a ratio of 2:1 (Crystal et al, 1984). In addition to collagen, the interstitial matrix includes elastin, proteoglycans, and fibronectins. The fibroblasts and interstitial connective tissue matrix are the structural framework that determine the alveolar shape and the mechanical properties of the lung during respiration (Crystal et al, 1984).

1.2. Interstitial Lung Diseases

The interstitial lung diseases are a heterogeneous group of disorders of the lower respiratory tract characterized by derrangements of alveolar wall structure and loss of functional alveolar capillary units as a consequence of inflammation, destruction and fibrosis (Crystal et al, 1984). Although more than 100 agents are known to cause interstitial disease, two thirds of all cases are of unknown etiology (Crystal et al, 1984). In many instances, the reparative process is chronic and relentless leading to destruction of the normal architecture, persistence of inflammatory cells in the alveoli and interstitium as well as increase in interstitial and intra alveolar fibrosis. Interstitial diseases result in severe abnormalities of gas exchange and functional impairment (Simon and Paine, 1995).

1.2.1. Idiopathic Pulmonary Fibrosis (IPF)

In most studies, IPF is the most frequently diagnosed cause of interstitial lung disease (Wolff and Crystal, 1997). IPF is a progressive and lethal disorder of unknown etiology that affects the alveoli and interstitium (Khalil and Greenberg, 1991). IPF is a process characterized by inflammation that involves the alveolar walls and the adjacent airspaces, release of fibrogenic mediators that result in the synthesis and accumulation of connective tissue (Crystal et al, 1984). The disease is progressive and results in death 3-6 years after the onset of symptoms. The prevalence is thought to be 5-23 cases/100,000 and the male/female ratio ranges from 1/1 to 2/1 (Coultas et al, 1994). The onset of the

disease usually occurs when patients are in their 50s or 60s, although children and elderly may occasionally be affected (Khalil and Greenberg, 1991).

The pathogenesis of IPF is poorly understood. It is speculated that there is an initial pulmonary injury in IPF that may be recurrent and may be the result of a variety of insults to the lower respiratory tract (Kelley, 1990). Alveolar macrophages may be important cells in regulating the inflammatory and fibrotic response associated with IPF. This is because alveolar macrophages increase in number at the time of injury and have been shown to produce a wide spectrum of fibroblast regulating cytokines (Kelley, 1990), such as PDGF, IL-1, TNF-α, bFGF and TGF-β (Khalil et al, 1991; McCartney et al, 1990; Shaw et al, 1991). Each of these cytokines not only regulates fibroblast function and collagen synthesis but also affects the recruitment and cytokine production of other inflammatory cells such as macrophages, neutrophils and lymphocytes (Kelley, 1990).

In pulmonary fibrosis the early stage of the disorder is characterized by a patchy alveolitis with mild to moderate thickening of the alveolar walls. As injury continues, the inflammation persists and there is progressive derangement of the alveolar structures, including loss of type I cells, proliferation of type II cells, migration of bronchiolar epithelial cells to the alveoli, loss of capillaries, and the thickening of the walls of small airways and arteries. The thickened interstitial matrix is expanded with large numbers of fibroblasts, myofibroblasts, smooth muscle cells and deranged fibers, particularly those made up of type I collagen (Crystal et al, 1984). With chronic and repeated cycles of injury and fibrosis there is extensive increase in lung collagen synthesis that results in

contraction of collagen on existing air spaces and formation of honeycomb cysts (Figure 2). Increased lung collagen synthesis in lungs has been demonstrated in several animal models of pulmonary fibrosis (Kehrer and Witschi, 1980; Snider et al, 1978; Starcher et al, 1978). In these instances the epithelial basement membranes are thickened and disruptions in the basement membrane occur, through which fibrosis passes into the air spaces.

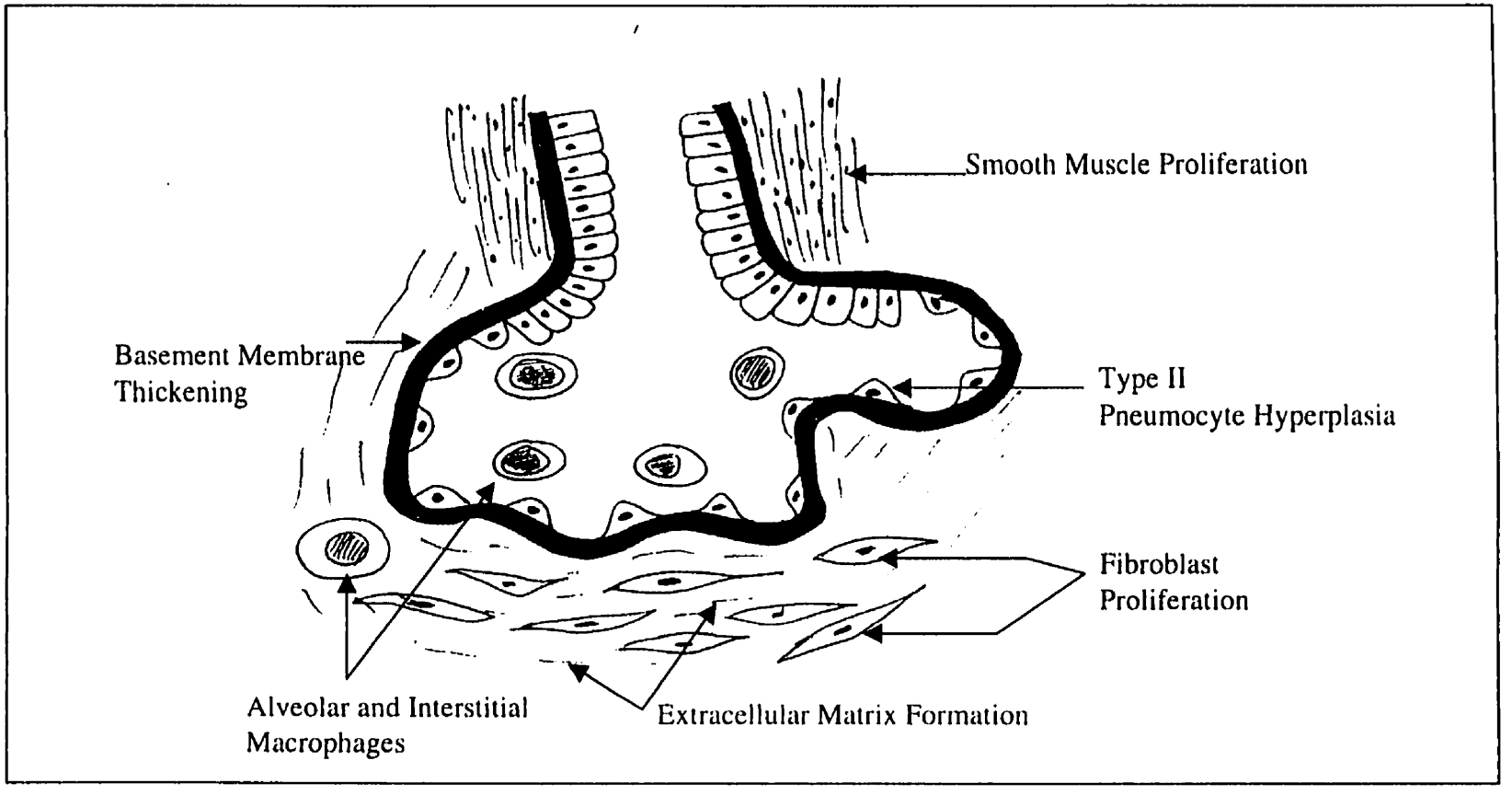
1.2.2. Models of Pulmonary Fibrosis

There are a number of agents that have been used to initiate pulmonary fibrosis in animal models including radiation, oxygen, paraquat, N-nitroso-N-methylurethane, bleomycin, viral infections, silica, asbestos, cadmium sulfate, ozone, bovine serum albumin, and anti lung serum (Hance and Crystal, 1975). Despite the agent used the general events in the lung are similar.

1.2.3. Bleomycin Induced Pulmonary Fibrosis

Bleomycin is an anti-neoplastic antibiotic that has been used over the last 10 years in treatment of several types of tumors, including squamous cell carcinomas, Hodgkins and non-Hodgkins lymphomas and testicular tumors (Umezawa et al, 1996). The limiting factor in prescribing bleomycin to patients is the risk of developing pulmonary fibrosis (Ingrassia et al, 1991). Bleomycin causes a dose-dependent pulmonary toxicity in animals, irrespective of the route of administration. The changes induced by bleomycin in

Figure 2: Honeycombing and Intersitial Fibrosis in Idiopathic Pulmonary Fibrosis



Reference: Khalil and Greenberg, 1991

animal models are the same as the changes seen in humans with lung toxicity to bleomycin (Adamson, 1984). In addition bleomycin induced fibrosis is similar to the early lesions in IPF lungs (Khalil et al, 1991).

Bleomycin-induced lung fibrosis is a complex process that involves cell-cell interactions, stimulatory and inhibitory factors, morphologic changes, immunologic changes, biochemical changes and the participation of many cell types (Breen et al, 1992). The most critical event following bleomycin administration is destruction of type I epithelial cells that line the walls of the alveoli. These cells are destroyed because they contain low levels of bleomycin hydroxylase, a detoxifying enzyme that cleaves the carboxyamide groups of bleomycin (Adamson, 1984; Lazo and Hoyt, 1990). In epithelial cells, once the drug crosses the cytoplasm into the nucleus, it inserts between the double helix and then cleaves the DNA, resulting in necrosis of the epithelial cells (Adamson, 1984). By destroying the epithelial barrier there is influx of inflammatory cells which is dominated by macrophages, neutrophils and lymphocytes (Simon and Paine, 1995). As the contents of the interstitium and blood leak through the damaged endothelium directly into the alveoli, aggregation of fibrin occurs. Fibrinogen, a normal constituent of plasma, becomes polymerized to fibrin when it enters the alveolar compartment during inflammation. Interstitial fibroblasts can then pass through gaps in the injured epithelium and move into the fibrin matrix. There they secrete collagen to produce areas of intra-alveolar fibrosis and a permanent increase in connective tissue (Adamson, 1984 ; Simon and Paine, 1995).

The direct intratracheal instillation of bleomycin into the lungs of adult rodents induces acute lung injury and fibrosis. After bleomycin administration the alveolar macrophages are activated to release a variety of cytokines such as platelet derived growth factor, interleukin-1, basic fibroblast growth factor, tumor necrosis factor alpha and transforming growth factor beta (Khalil et al, 1996).

1.3. Cytokines in Tissue Injury

Cytokines are extracellular signaling proteins secreted by specific cells (Kelley, 1990). These molecules function primarily to modify the behavior of other closely adjacent cells. The short extracellular distances over which cytokines travel before interacting with target cell receptors distinguish them from the circulating endocrine hormones (Kelley, 1990). A central event in tissue repair is the release of cytokines in response to injury (Border and Noble, 1994). These cytokines, in conjunction with other mediators are important regulators of cell functions that are of direct relevance to the fibrotic response (Phan, 1994). The cytokines demonstrated to be important in pulmonary fibrosis are platelet-derived growth factor, basic fibroblast growth factor, tumor necrosis factor, interleukin-1 and TGF-β (Kelley, 1990).

1.3.1. Transforming Growth Factor-Beta

Of the cytokines induced during lung injury TGF-β is one of the most potent regulators of inflammation and fibrosis. Transforming growth factor beta represents a prototype of a large superfamily of growth, differentiation, and morphogenesis factors, all of which have been shown to be structurally related to TGF-β (Massague, 1990). The

TGF-β superfamily comprises more than 30 distinct members that are grouped in several subfamilies, including the TGF-β family, the activin/inhibin family, bone morphogenetic proteins, the Drosophila decapentaplegic gene product, Mullerian inhibiting substance, the Xenopus Laevis Vg-1 and Vgr-1 and the glial cell line derived neurotrophic factor (Alevizopoulos and Mermod, 1997; Sporn and Roberts, 1992). The distribution of TGF-β related factors is conserved throughout evolution in organisms ranging from fruit flies to humans (Albright and Oppenheim, 1991; Massague, 1990). A high degree of conservation, 99% between human and mouse TGF-β_1 sequences, supports a critical biological role of the TGF-β's across species (Massague, 1987).

The TGF-β family consists of at least five genes encoding distinct proteins in vertebrates referred to as TGF-β_{1-5}. (Alevizopoulos and Mermod, 1997). Mammals express three isoforms of TGF-β designated TGF-β_1, TGF-β_2 and TGF-β_3 (Massague, 1990; Sporn and Roberts, 1992). The gene for each isoform is located on a different chromosome and differential expression may occur through distinct cell-specific regulatory mechanisms (Wahl 1992). The transcriptional regulation of TGF-β_{1-3} is distinct due to their unique promoter regions and 5′ and 3′ untranslated regions. TGF-β_1 expression is selectively induced in response to a variety of stimuli following wounding, ischemia, carcinogenesis and fibrogenesis. TGF-β_2 and β_3 expression is regulated primarily in response to hormonal and developmental signals (Roberts et al, 1996).

Relevant to lung tissue injury and fibrosis is that TGF-β_1 is the most abundant isoform in human platelets, the first cellular element to arrive at a site of injury. Platelets

represent the most concentrated natural source of TGF-β_1, producing nearly 20 mg/kg (Wahl, 1992). The immediate release of TGF-β by platelets at the site of injury indicates that TGF-β might play a role as a mediator of the inflammatory response (Roberts et al, 1986; Wahl, 1992). This release of TGF-β_1 is responsible for recruitment of inflammatory cells at the site of injury so the repair process can begin. TGF-β_1 isoform has been described to be the most prevalent isoform in areas of injury and fibrotic diseases. For example, more than 85% of TGF-β in adult wound fluid is the TGF-β_1 isoform (Roberts et al, 1996). In addition TGF-β is the most potent stimulator of chemotaxis *in vitro* (Wahl et al, 1987) and *in vivo* (Roberts et al, 1986). It can stimulate the migration of monocytes in fentomolar concentration range (Wahl et al, 1987). Furthermore, monocytes and macrophages are not only increased at sites of injury but are also an important source of TGF-β_1 (Khalil et al, 1989). Since TGF-β_1 can induce monocytes/macrophages to generate PDGF, IL-1, TNF-α and itself, the presence of TGF-β at the site of injury would result in further augmentation of macrophage dominated infiltrate and generation of fibrogenic cytokines. The effects of TGF-β_1 on fibroblasts are crucial in wound healing. Fibroblasts are responsive to the chemotactic action of TGF-β even when present in femtomolar concentrations (Postlethwaite et al, 1987). At higher concentrations TGF-β_1 is no longer chemotactic and acts to augment the expression of genes of the fibroblasts that encode intercellular matrix proteins and simultaneously decreases proteolysis of the intracellular matrix (Albright and Oppenheim, 1991). (Varga et al, 1987) showed that TGF-β may play an important regulatory role in fibrogenesis. They demonstrated a several fold increase in the production of collagen I, collagen II and fibronectin by normal human dermal fibroblasts in response to TGF-β_1. The

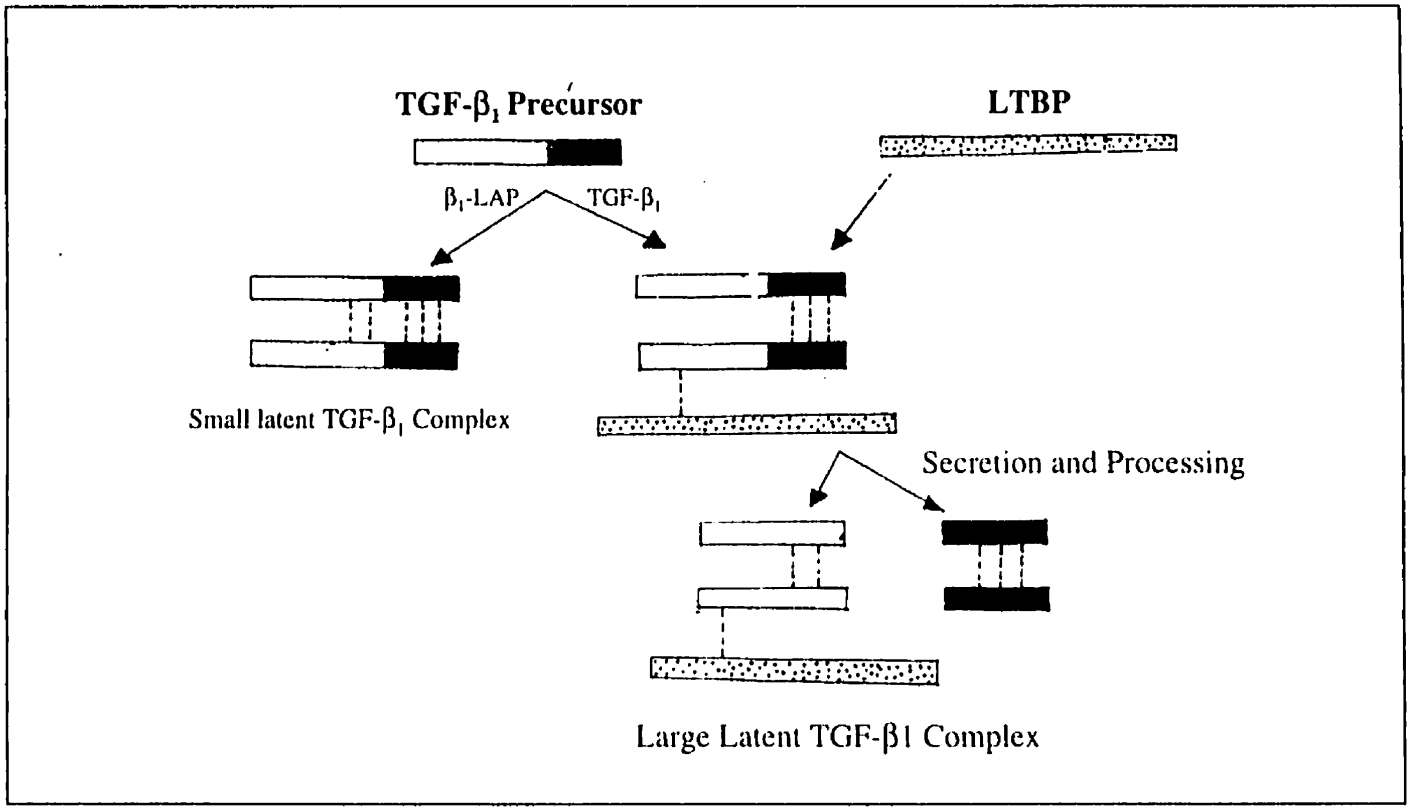
corresponding mRNA's of the connective tissue proteins remained elevated 72 hours after removal of TGF-β_1, indicating a stimulatory role of TGF-β_1 on connective tissue production in fibroblasts. Furthermore, the synthesis of inhibitors of degrading enzymes such as plasminogen activator inhibitor of metalloproteinases are also increased by TGF-β, while the expression of proteases such as collagenase, transin/stromelysin and plasminogen activator are decreased (Attisano et al, 1994; Roberts et al, 1990). It is speculated that once cells have reached the zone of higher TGF-β_1 concentration, both cell migration and proliferation become inhibited, so that the cellular behavior is dominated by functions of differentiation (Moses et al, 1990). Human lung biopsies obtained from eight patients with IPF showed elevated levels of TGF-β_1 expression at sites of active fibrosis. (Khalil et al, 1991) demonstrated that intense staining for TGF-β_1 was localized to sites where increased numbers of fibroblast were present. Their results also demonstrated that TGF-β_1 in areas of increased fibroblasts was present in close association with extracellular matrix suggesting that TGF-β associated with fibroblasts may serve to increase synthesis and deposition of extracellular components such as fibronectin and some forms of collagen. In addition to IPF, TGF-β_1 has been shown to be expressed in increased quantities in animal models and in tissues of several other human diseases that are characterized by inflammation and fibrosis such as glomerlonephritis in the kidney (Okuda et al, 1990), Schistosomiasis (Czaja et al, 1989), liver cirrhosis (Castilla et al, 1991; Nagy et al, 1991), cardiac fibrosis after infraction, scarring and disorders of the eye and skin, as well as in the formation of postoperative fibrosis intra-abdominal adhesions (Border and Ruoslahti, 1992).

The other isoforms of TGF-β are more ubiquitously expressed and less often associated with injury. The distribution of TGF-β_2 is principally found in aqueous and vitreous humor of the eye (Connor et al, 1989), amniotic fluid (Altman et al, 1990), saliva and breast milk (Jin et al, 1991), suggesting a physiological rather than pathological function. All the TGF-β isoforms are 25 kDa disulfide-linked dimers that share more than 70% sequence homology at the amino acid level (Roberts et al, 1996).

1.3.2. Post-Translational Activation of Latent TGF-β

All isoforms of TGF-β are invariably secreted from cells in a form that is unable to bind and activate its signaling receptors and are called latent TGF-β (Massague, 1990; Roberts et al, 1996). Two forms of latent TGF-β have been described and are termed the small and large latent complexes (Figure 3). The small latent complex is derived from a single gene product and consists of a noncovalent complex between the mature C-terminal 112 amino acid TGF-β and the N-terminal remainder of its own pre-pro domain (residues 30-278), called the latency associated peptide (LAP) (Roberts et al, 1996). TGF-β is biologically inert when it is in its latent form. Since TGF-β and its receptors are so ubiquitous and the biological effects so varied, an important mechanism by which biological effects are controlled are by regulation of the post-translational activation of LTGF-β_1. The latent form of TGF-β has a half life of greater than 90 minutes (Wahl, 1992). Mechanisms by which TGF-β can be activated include exposure to extreme pH (<4 or >9), chaotropic agents (sodium dodecyl sulfate, urea), heat and proteases such as plasmin and (TSP-1) thrombospondin-1 (Massague, 1990). There may be several *in vivo* mechanisms of activation of LTGF-β. For example, activation of LTGF-β occurs by

Figure 3: Biosynthesis and Processing of LTGF-β



Reference: Roberts et al, 1996

anchoring the molecule to activation sites on the cell surface or extracellular matrix via latent TGF-β_1 binding protein (LTBP) and/or via binding of mannose-6-phosphate residues on LAP to the mannose-6-phosphate receptor (Roberts et al, 1996). Recently, our laboratory has described that alveolar macrophages activated *in vivo* by bleomycin injury, generate LTGF-β_1-TSP-1 complex that is localized to the cell surface by interacting with the TSP-1 receptor CD36 (Yehaulaeshet, submitted). Upon localization to the cell surface, plasmin then cleaves the mature peptide (Khalil et al, 1996). When LAP is covalently linked to another protein called the latent TGF-β binding protein (LTBP) (Roberts et al, 1996) the entire complex is called large latent TGF-β. When LTGF-β is complexed with LTBP, TGF-β can associate with a variety of matrix proteins including biglycan, decorin, type IV collagen, fibronectin, and thrombospondin. The ability of LTGF-β-LTBP complex to interact with extracellular matrix proteins may be important in storing an inactive form providing a reservoir of the peptide. (Roberts et al, 1996).

1.3.3. Effect of TGF-β on Extracellular Matrix Synthesis

The extracellular matrix plays an important supporting structural role in the lung (Roberts et al, 1990). It continuously changes shape and volume. The effect of TGF-β_1 on resident cells which produce extracellular matrix is central to understanding the pathological matrix accumulation that is characteristic of fibrotic diseases (Roberts et al, 1990). TGF-β_1 has been shown to induce the production of a wide variety of proteins found in the extracellular matrix including type I, II, III, IV, and V collagen, thrombospondin, osteopontin, tenascin, elastin, hyaluronic acid, osteonectin/SPARC, as

well as chrondroitin/dermatan sulfate proteoglycans such as biglycan, and decorin (Roberts et al, 1992). The direct effect of TGF-β_1 on synthesis of these components is accompanied by its ability to interfere with proteolytic degradation of matrix proteins (Roberts et al, 1990). This occurs by reducing synthesis and secretion of different proteases that act on the extracellular matrix and by increasing synthesis of specific inhibitors to those proteases. In addition to its effects on extracellular matrix protein deposition, TGF-β_1 also increases cellular expression of the integrin family of adhesion protein receptors (Ignotz et al, 1989). These are glycoproteins that bind extracellular matrix components to the cell membrane. TGF-β increases levels of receptor mRNA and the rate of processing of the receptor subunits by cells, suggesting that TGF-β can modulate the ability of a cell to adhere to extracellular matrix components (Roberts et al, 1990).

Pathological accumulation of matrix may result not only from excessive production of TGF-β_1 but also from defects in the signaling pathways emanating from matrix that would ordinarily function to terminate or suppress expression of TGF-β_1 (Nathan and Sporn, 1991). This would imply a bi-directional interaction between TGF-β and the extracellular matrix.

1.3.4. Control of Cell Phenotype

Other functions of TGF-β that may be important in wound repair are regulation of proliferation and induction of differentiation. Epithelial cells and fibroblasts are examples of cell types in which TGF-β has regulated proliferation and differentiation.

Although *in vitro* and *in vivo* TGF-β_1 is inhibitory to epithelial cell proliferation (Jetten at al, 1986), during wound repair when there is reepithelialization, TGF-β_1 stimulates differentiation of type II epithelial cells to type I epithelial cells (Khalil et al, 1994). TGF-β_1 has also been shown to stimulate proliferation of immature fibroblasts (Hill et al, 1986), implying that the presence of TGF-β_1 at the site of injury could expand the population of fibroblasts. This is very important in the context of wound repair because TGF-β_1 induces fibroblasts to synthesize connective tissue proteins like collagen and fibronectin (Khalil et al, 1989).

1.3.5. TGF-β is both Proinflammatory and Immunosuppressive

TGF-β has been demonstrated as a bifunctional peptide having both immunosuppressive and potent pro-inflammatory roles. These opposing effects are controlled by the cytokine's selective production and latency, as well as receptor modulation and differential susceptibility of target cells at various stages of development, maturation and activation (Wahl, 1992).

TGF-β is immunosuppressive because it has been shown to inhibit both cellular and humoral responses (Ellingsworth et al, 1988; Saski et al, 1992). This includes inhibiting proliferation of thymocytes in response to IL-1, inhibiting proliferation of T cells in response to IL-2, inhibiting B cell responses to growth factors as well as secretion of Igs by Ig producing cells, and inhibiting the generation of lymphokine-activated killer cells and cytotoxic T cells (Albright and Oppenheim, 1991; Fontana et al, 1992). Wahl

(1992), speculate that such opposing effects of TGF-β on cells arise at different times in their life cycle; TGF-β is released early and functions to promote inflammation based on its effects on immature monocytes and T cells. Once these cells differentiate and become activated, they become susceptible to phenotypic modulation and functional inhibition by TGF-β.

1.3.6. Transforming Growth Factor Beta Receptors

TGF-β interacts with three highly conserved receptors designated TGF-β receptors I, II, and III often abbreviated as TβR-I, TβR-II and TβR-III (Attisano et al, 1994). TβR-I is a 50-60 kDa while TβR-II is 75-80 kDa and TβR-III is 280-330 kDa (Yingling et al, 1995). Both type I and type II TGF-β receptors are transmembrane serine/threonine kinases while TβR-III has no signaling domain (Attisano et al, 1994). TGF-β elicits its biological effects by signaling through a heteromeric receptor complex consisting of the type I and type II TGF-β receptors. Binding of TGF-β to the type II receptor results in the recruitment of the type I receptor into a signaling complex (Wrana et al, 1994; Yamashita et al, 1994). As a result of its recruitment into the complex, the cytoplasmic domain of the type I receptor is phosphorylated on serine and threonine residues (Wrana et al, 1994). Phosphorylation of the type I receptor is mediated by the kinase activity of the type II TGF-β receptor (Wrana et al, 1994). This phosphorylation enables the type I receptor to activate signaling cascades that lie downstream of the TGF-β receptors. In summary, receptor I requires receptor II for ligand binding and receptor II requires type I to generate a TGF-β induced signal.

1.3.7. Distribution of TGF-β Isoforms in Lung Injury and Repair

Immunohistochemical studies have shown that the three isoforms are expressed in unique patterns following excisionl wounding (Levine et al, 1993; Roberts et al, 1996). In bleomycin-induced pulmonary fibrosis in the rat and human fibrotic lung diseases including IPF, immunohistochemical staining demonstrates that TGF-β_2 and TGF-β_3 are ubiquitously expressed in both normal and fibrotic lungs (Khalil et al, 1996). However, after bleomycin injury alveolar macrophages are almost exclusively the source of TGF-β_1 in early pulmonary lesions found in rat lungs (Khalil et al, 1989, 1993, 1996). In human patients with advanced pulmonary fibrosis and honeycombing, TGF-β_1 has been shown to be expressed in epithelial cells, macrophages, and associated with extracellular matrix (Khalil et al, 1996). Since TGF-β_1 is not normally expressed in alveolar epithelial cells, the TGF-β_1 expression in epithelial cells of fibrotic lungs may be an indication of the chronicity of injury. These findings prompted us to investigate whether the production of TGF-β_1 by alveolar epithelial cells is important in the inflammation and fibrotic changes seen in advanced lung injury. We hypothesized that if we overexpress TGF-β_1 in epithelial cells this will lead to increased localized inflammatory changes and connective tissue synthesis. To overexpress TGF-β_1 in alveolar epithelial cells we chose to design retroviral vectors containing the cDNA for TGF-β_1 and transfect the alveolar epithelial cells *in vivo*.

1.4. In vivo Transfection with Genetic Material

One of the most current areas of research is insertion of genetic material into eukaryotic cells for the purposes of overexpression of a protein of interest. This is

achieved by using a vector as a gene delivery tool. Two general categories of gene transfer vectors have been developed to improve the efficiency of delivery into mammalian cells. They are viral and non-viral vectors (Curiel et al, 1996; Roth and Cristiano, 1997).

1.4.1 Non-viral Vectors

There are a variety of physical and chemical methods of introducing genes into cells. Physical methods for the introduction of DNA into cells include microinjection, particle bombardment and electroporation (Neumann et al, 1982). Although direct injection of DNA into tissue or high velocity bombardment of tissues with DNA attached to gold particles is reasonably efficient, this method of delivery is limited to the injection site with no tissue targeting. (Cheng et al, 1993; Nabel et al, 1991). An alternative physical approach is electroporation. This method involves transport of DNA into the cell by disrupting the surface membrane with a rapid pulse of high voltage current (Neumann et al, 1982). Exogenous DNA can also be introduced into cells by chemical methods, including co-precipitation with calcium phosphate and incorporation of DNA into liposomes (Nabel et al, 1992; Roth and Cristiano, 1997). Lipofection is a method that involves DNA to be incorporated into artificial lipid vesicles delivering their contents directly into the cell cytoplasm. This method is efficient because the synthetic spheres fuse with the cell membranes (Watson et al, 1992).

1.4.2. Retroviral Vectors

The most useful vectors for the efficient introduction of foreign genes have been derived from murine and avian retroviruses (Nabel et al, 1991). A vector is constructed by initially deleting three structural genes required for viral replication. The gag gene encodes group specific antigens, pol encodes reverse transcriptase and integrase, and env encodes the envelope protein (Miller, 1990; Temin, 1989). Forgein DNA for the gene of interest is ligated into the deleted genome of the virus which retains a sequence required for viral packaging. The replication-defective retroviral vector is introduced into a packaging cell line into which gag, pol, and env genes devoid of packaging signals have been previously transfected (Danos and Mulligan, 1988). The structural genes are provided to the retroviral vector from within the packaging cell to allow for the production of virus particle containing the foreign gene of interest (Watson et al, 1992).

The principal advantage of this type of vector is its ability to accomplish efficient and stable gene transfer in a broad range of targeted cells as well as its capacity to achieve integration into the host genome (Kotani et al, 1994; Varmus, 1988). However a number of restrictions also apply to the use of retroviruses. The packaging capacity of the virion is limited up to 6 kb, the requirement that the cell being transfected be proliferating and *in vivo* lability has severely limited the utility of these agents (Miller et al, 1990). Lastly, standard retroviruses can be produced only in low titers (number of infectious viral particles in a given volume) (Curiel et al, 1996).

To overcome this limitation, strategies have been developed to generate high titer retroviral vectors (Kozak and Kabat, 1990; Ruden et al, 1995). The "ping pong" method uses the principal that infection of packaging cells with a virus results in higher titers than can be obtained by transfection of packaging cells. Secondly, co-cultivation of two packaging lines producing viruses of different host range may lead to enormous increases in virus production as a consequence of a marked increase of the proviral copy number. (Bestwick et al, 1988 ; Lynch and Miller, 1993).

1.4.3. Adenoviral vectors

Recombinant adenoviral vectors are the most widely employed vectors for gene transfer to the lung (Munaf et al, 1994 ; Curiel et al, 1996). A major advantage is that unlike retroviruses, adenoviruses can be concentrated to produce high titers (up to 10^{12}) CFU/ml. They are also able to accomplish gene delivery to terminally differentiated cells (Curiel et al, 1996).

The disadvantage of adenoviruses relate to properties of the vector and host response. The adenoviral genome is also unable to integrate into the host chromosome and thus gene expression is only transient. Further more, when adenoviruses are used for direct *in vivo* application, the virons elicit a prominent inflammatory response (Curiel et al, 1996 ; Ye et al 1994).

2. Project Objectives

To determine if the aberrant production of biologically active TGF-β_1 by alveolar epithelial cells *in vivo* regulates an inflammatory and fibrotic process.

Hypothesis: If we overexpress TGF-β_1 in epithelial cells using a retrovirus, this will lead to increased localized inflammatory changes and connective tissue synthesis.

Experimental Approach

1) Package the retroviral vectors into infectious particles.
2) Titer the viral particles in CFU/ml.
3) Infect a cell line to test the promoter inducibility and quantitate gene expression.
4) Purify the viral particles and suspend in a non-immunogenic vehicle.
5) *In vivo* transfection using retroviruses.

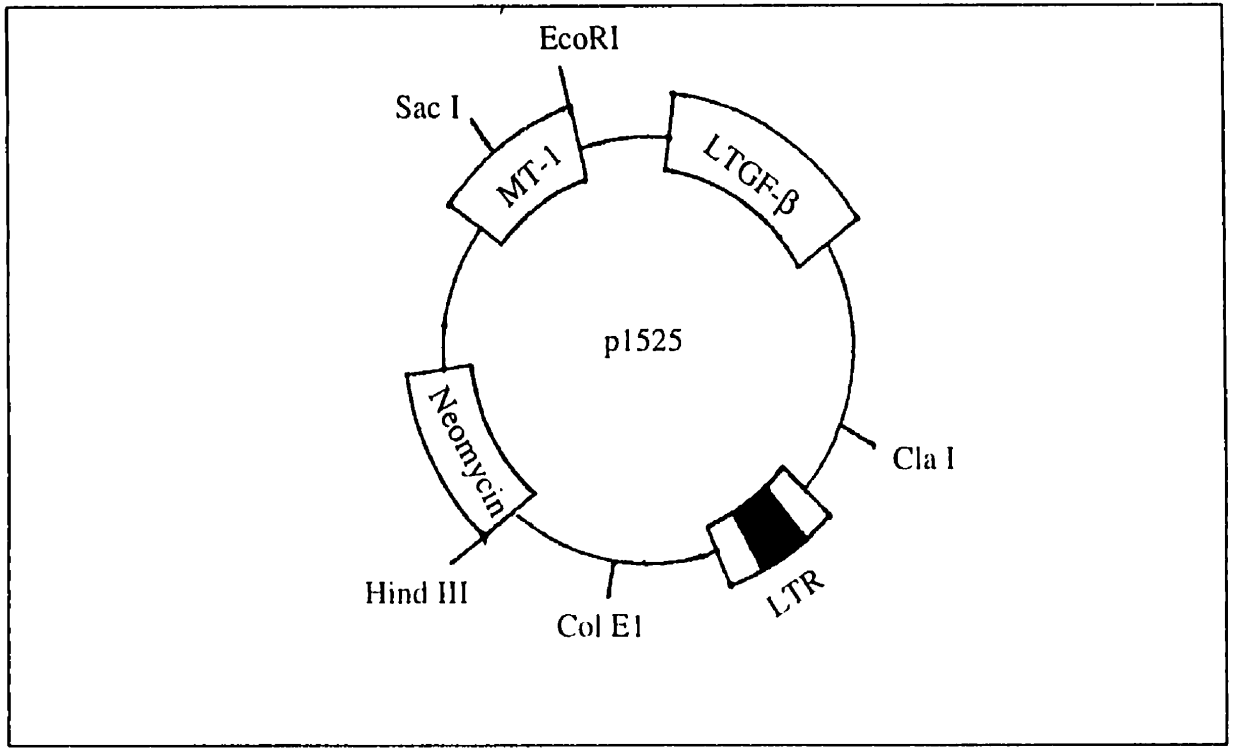
3. Materials and Methods

3.1 Retroviral Vectors

3.1.1. p1525

The plasmid p1525 carries the LTGF-β_1 cDNA (gift from Dr. Mary Lou Cutler, NIH) that is 1.2 kb long. It is controlled by a zinc inducible metallothionine promoter. The plasmid carries a geneticin drug marker gene and is flanked by LTR regions and also has a packaging signal (Figure 4).

Figure 4: Map of Retroviral Vector Carrying LTGF-β_1 cDNA



3.1.2. p1521

The plasmid p1521 (gift from Dr. Mary Lou Cutler, NIH). It is exactly like p1525 except it contains no TGF-β insert (Figure 5).

3.1.3. p1521/TGF-β

The retroviral vector contains the cDNA for TGF-β_1 that encodes for active TGF-β_1. This is because the Cys 223 and Cys 225 amino acids were mutagenized by converting them to serine residues, resulting in a latency associated peptide (LAP) that does not remain associated with the mature TGF-β_1 upon secretion. The p1521/TGF-β_1 retroviral vector was constructed by restriction digesting a cDNA for porcine active TGF-β_1 out of a pPK9A vector (gift from Dr. Greenberg, Manitoba Institute of Cell Biology) and ligating it into the EcoRI site of the retroviral vector p1521 (Figure 6).

Figure 5: Map of Empty Retroviral Vector

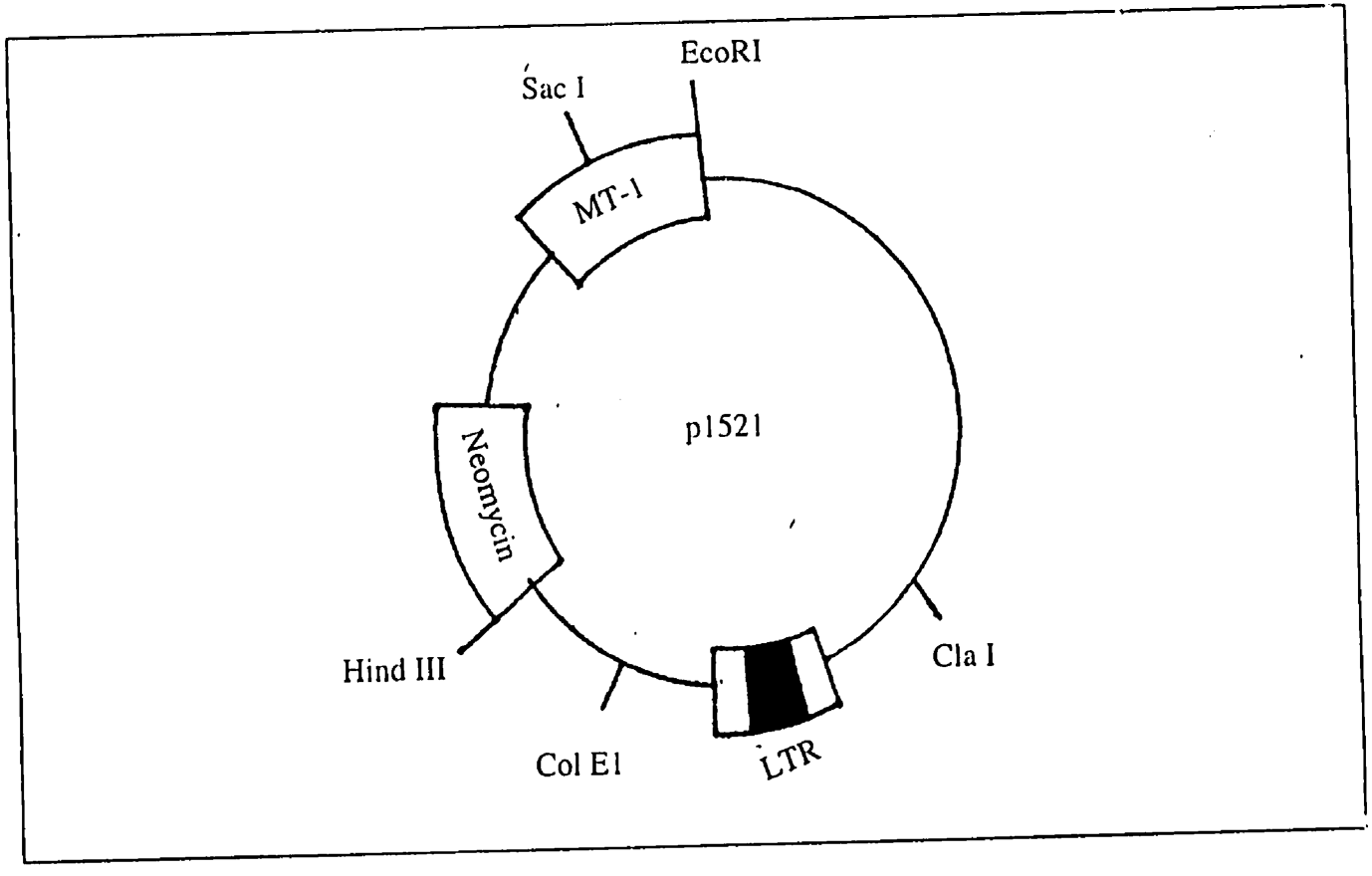
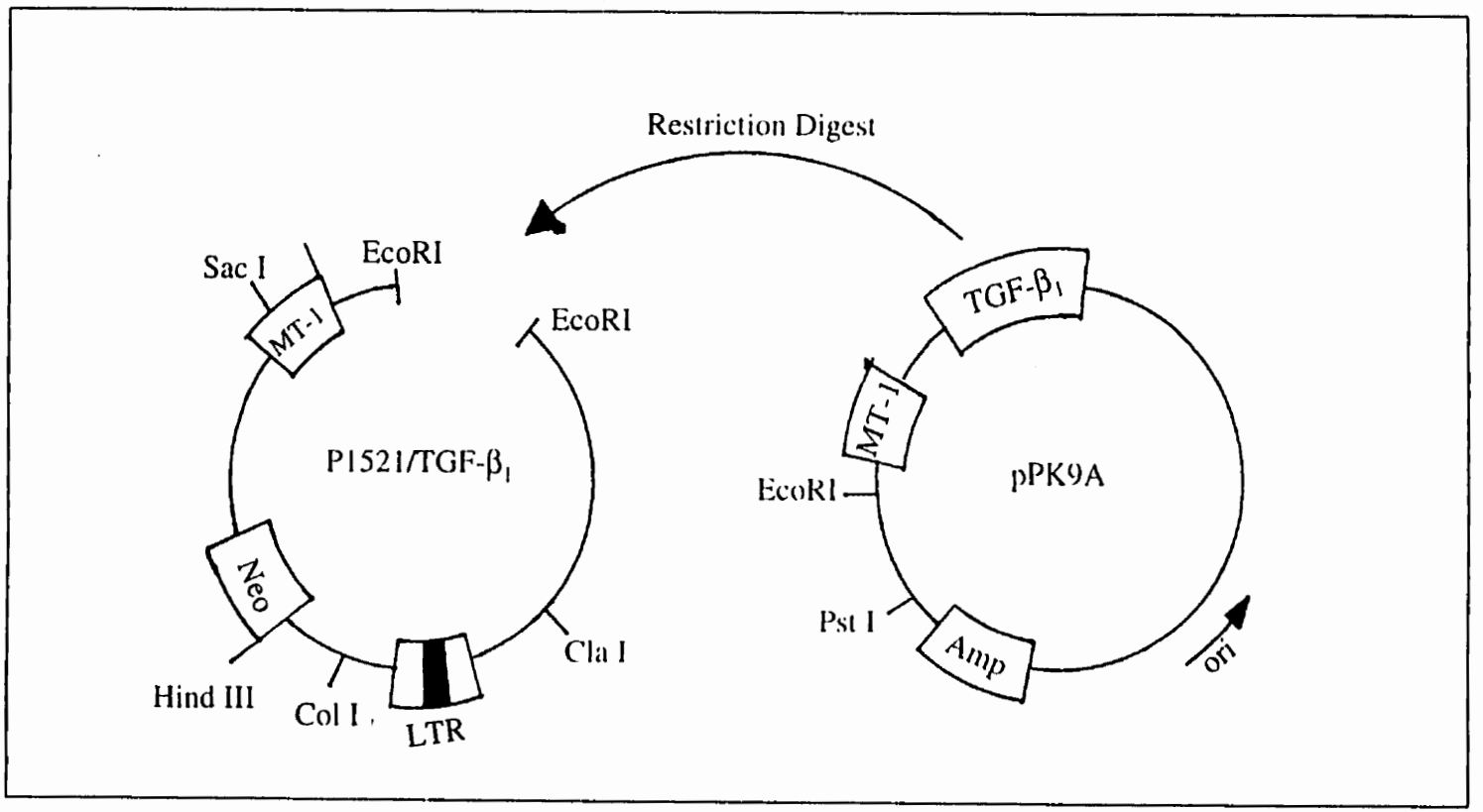


Figure 6: Map of Retroviral Vector Carrying Bioactive TGF-β_1 cDNA



3.2 Molecular Methods

3.2.1 Bacterial Transformation

A DH5α strain of competent E.coli bacteria were transformed by electroporation with plasmids p1521, p1525, p1521/TGF-β, and pCMV/TGF-β (Table 1). In pre-chilled 1.5 ml microfuge tube containing DH5α competent bacteria, 40ng of plasmid DNA was mixed in by tapping the tubes. The contents of the microfuge tube were transferred into pre-chilled cuvettes and placed in the electroporation chamber. An electrical pulse of 420 ohms was applied for 20 milliseconds to transform the bacteria. Immediately after the transformation, the bacteria were washed out of the cuvettes with 1 ml of SOC medium (mixture of tryptone, yeast extract, NaCl, KCl, glucose, and 2 M magnesium) and transferred to a sterile culture tube with a pasteur pipette. The culture tubes were incubated for 60 minutes in a shaking 37 °C incubator. Aliquots of 200 µl were spread with a glass rod onto LB (Luria-Bertani Medium) plates containing the appropriate antibiotics (Table 1). The plates were incubated face down in a 37 °C incubator overnight and checked for colonies the next day.

Table 1 : Summary of conditions used for transformation of DH5α

Plasmids	p1521	p1525	p1521/TGF-β	pCMV/TGF-β
	50 µg/ml	50 µg/ml	50 µg/ml	----------
	---------	----------	----------	60 µg/ml
	~ 90	~ 75	~ 50	~ 60

3.2.2 Plasmid Isolation and Purification

A small scale plasmid purification was carried out on single colonies using the Wizzard kit (Promega). Overnight cultures were centrifuged for five minutes at 4°C at 6000 x g (Sorvall GSA). The pellets were suspended in 200µl of cell resuspension solution. Then 200µl of cell lysis solution was added and the tubes were mixed by inverting several times. The mixture was neutralized and all the tubes were centrifuged at 13,000 rpm for 5 minutes. The supernatant was passed through a resin in a minicolumn provided by the kit. The columns were washed and the plasmid DNA was eluted with 50 µl of sterile water into 1.5 ml microfuge tube.

Restriction digests were set up using the appropriate restriction enzymes for all the picked clones (Table 2) to confirm the presence/absence of insert. The reaction mixture was made up using 6µl plasmid, 1.5ul 10x buffer, 1.0 µl enzyme, 2.5µl sterile water. The mixture was incubated at 37° C for three hours, the samples were subjected to electrophoresis on 1% agarose gel (electrophoretic grade agarose, 1X TBE) at 60 v for 1-2 hours. A Lambda HindIII marker was run beside the digested samples to determine fragment size. A photograph was taken for a permanent record of clones containing the appropriate sized insert.

Clones with the appropriate inserts were selected for large scale plasmid preparations. Aliquotes of 2 ml LB broth containing the appropriate concentration of antibiotics was inoculated with bacteria. These initiating cultures were incubated with shaking containing antibiotics and incubated overnight at 37° C with shaking. The

plasmid was purified using the Qiagen Plasmid Kit (Qiagen Inc.) according to protocol without modification. A restriction digest as outlined above was repeated on these purified samples to determine quality of the purification procedure and yield of the insert. A photograph was taken for permanent record of the two clones containing the appropriately sized insert.

Table 2: Summary of Clones with Correct Band Size

Plasmid Type	p1521	p1525	p1521/TGF-β	pCMV/TGF-β
	6	9	12	15
	6	5	4	8

3.2.3 Constructing Infectious Particles

Retroviral vectors are packaged into infectious particles using special packaging cell lines. In our lab the ψ_2 packaging cell line was used. The ψ_2 cells were grown on 100 mm tissue culture plates in αMEM media with 10% FBS for 2-3 days until confluent. For each vector transfection, 1.0×10^5 ψ_2 cells were plated in 100 mm plates and left in a 37°C incubator in the presence of 5% CO_2 in a humidified atmosphere over

night before calcium phosphate transfection the next day.

In 1.5 ml microfuge tubes 10 μg of plasmid DNA was diluted into 200 μl of sterile water; control plates contained only 200 μl of sterile water. To each tube 750 μl of 2X HeBS (hepes buffered saline) pH 7.05 was added and then drop by drop 50 μl of 2.5 M $CaCl_2$ was mixed in. The tubes were allowed to sit for 30 minutes at room temperature. The plated ψ_2 cells from the night before were taken out of the incubator and the calcium phosphate DNA precipitates were added to the ψ_2 cells. The plates were incubated overnight in 37°C incubator and the next day media was replaced with fresh media with 400 μg/ml geneticin (G418) for selection of clones. Individual clones were identified and marked on the bottom of the plates with a marker. Cells that were not transfected died from the drug selection. Over the next few weeks the stably transfected clones were transferred to 60 mm tissue culture plates until they were confluent and then they were finally transferred to 100 mm plates. Once the 100 mm plates became subconfluent, the media was replaced with fresh media and the next day the about 9 ml of viral supernatant was collected with a 10 cc syringe, filtered with 0.2 μm filter and was frozen in 1.5 ml vials in a -80° C freezer. The transfected ψ_2 cells were allowed to grow in the presence of geneticin until they were confluent at which point they were trypsinized, spun down and their pellets were resuspended in 1 ml 95% FBS αMEM, 5% DMSO and frozen in -80° C. The pCMV/ ATGF-β vector was not packaged because it is not a retroviral vector. This vector was calcium phosphate transfected into NIH 3T3 cells and clones were isolated after selection with geneticin.

Table 3: Summary of Number of Clones for each Plasmid

Plasmids	p1521	p1525	p1521/TGF-β	pCMV/TGF-β
	14	5	5	4

3.2.4. Ping Pong Amplification

Ping Pong amplification is cross infection of virus between two different packaging cell lines (Kozak and Kabat, 1990). This is a successful way of increasing the copy number of the gene of interest because envelope protein produced by one type of packaging cell line is different from another type and reinfection of cells is possible. The second type of packaging cell line we used is the PA317 line.

ψ_2 clones that produced the highest titer were picked for ping pong amplification. The ψ_2 transfected clones that had been previously frozen in the -80° C freezer were plated in 100 mm tissue culture plates in 10% FBS α MEM media until they became subconfluent. At the same time PA317 cells were seeded at $1.0x10^5$ cells per 100 mm tissue culture plate overnight. For cross infection to occur 1 ml of virus collected from the ψ_2 tranfected clones was added to the PA317 cells in the presence of 8 μg/ml polybrene. The polybrene was added to make the cells more permeable to the virus particles. The PA317 cells were incubated in the presence of geneticin in a 37° C incubator for a few weeks until the cells became subconfluent and virus was collected in the same manner already described for the ψ_2 cells. The cell lines were cross infected by

taking virus collected from the PA317 cells and re-infecting the already transfected ψ_2 cells. After a week with geneticin selection, 1 ml of virus was collected from the ψ_2 cells and the PA317 cells were re-infected. Each round of cross infection between the two cell lines produced packaging cells with increased gene copy number. After 1-3 rounds of cross infections the two packaging cell lines were frozen down in -80 °C. The idea behind using this method was to increase copy number of the gene so that protein expression could be maximized.

3.2.5 Titration of Viral Particles

In order to quantitate the amount of viral particles present in the supernatant it is necessary to infect a cell line and determine the colony forming units present per ml (CFU/ml) of supernatant.

In our lab NIH 3T3 mouse fibroblasts were available. The cells were seeded at $1.0x10^5$ cells in 100 mm tissue culture plates with 10% FBS αMEM and incubated at 37°C overnight. The next day the medium of the cells to be infected was replaced with fresh media and 8μg/ml polybrene was added to the plates. Virus was quantitated by adding 1 ml of a 1/10, 1/100, and 1/1000 serial dilution of viral supernatant to each plate. The media was again replaced the following day to wash away the polybrene because it is toxic if left too long on the cells. Over the next few weeks the cells were growth selected with 400 μg/ml of genetic. The cells that did not take up the virus were killed and individual clones were allowed to grow. The plates were stained with crystal violet, rinsed and allowed to dry. The number of colonies from the 1/10, and 1/100 serial

dilution's were too numerous to count. The 1/1000 serial dilution was used to calculate the CFU/ml. Five clones for each plasmid were titered and the colony forming units for each was determined. These titers are after ping pong amplification.

Table 4: Summary of Clones with Highest Titers

Plasmids	p1521	p1525	p1521/TGF-β
	$9.5x10^4$	$1.0x10^5$	$1.5x10^5$

3.2.6 Collecting Conditioned Media

Infectivity was tested on the highest titered viral particles by infecting NIH 3T3 cells. The cells were plated at a density of $1.0x10^5$ in 100 mm plates in 10% FBS α MEM. Next day 1 ml of viral particles was added to each plate in the presence of 8 μg/ml polybrene. Over the next few weeks the cells were growth selected with 400 μg/ml of geneticin. Once the 100 mm plates with the stably infected NIH 3T3 cells became confluent, the cells were plated out to collect conditioned media. Each 60 mm tissue culture well was seeded with $2.5x10^5$ NIH 3T3 uninfected cells and NIH 3T3 virus infected cells in 0.2% BCP. The plates were placed in a 37° C incubator for 2-3 hours so that the cells would have time to adhere. Then the cells were treated in the presence/absence of 100 μM zinc sulfate to induce the metallothionine promoter that controlled TGF-β gene expression. Media was collected at 24 and 48 hours in the presence of 0.5 μg/ml leupeptin, 1μg/ml aprotinin, and pepstatin and then aliquoted and

frozen at -80°C.

3.2.7 CCL 64 Mink Lung Epithelial Growth Inhibition Assay for TGF-β

The CCL 64 mink lung epithelial growth inhibition assay is a very sensitive biological assay that quantitates fentomolar quantities of TGF-β present in a sample by the extent of growth inhibition the cells experience. The CCL 64 mink lung epithelial cells were maintained in αMEM with 10% FBS. Cells were trypsinized and washed with αMEM, 0.2% BCP and resuspended in αMEM, 0.2% BCP and cultured as $4.5x10^4$ cells/ 0.5 ml in 24 well tissue culture plates. The cells were incubated for 2-3 hours at 37°C, at which time neutral conditioned or conditioned media that was acidified and subsequently neutralized was added to the wells. The plates were incubated overnight in a 37°C incubator. Next day they were pulsed with 0.25 μCi of 5-[^{125}I] iodo 2'-deoxyuridine for 2-3 hours at 37°C. Cells were then fixed with 1 ml of methanol-acetic acid (3:1) (vol/vol). After 1 hour at room temperature, the wells were washed twice with 2 ml of 80% methanol. The cells were lysed with 1 ml of 1 N NaOH for 30 minutes at room temperature and the 5'-[^{125}I] iodo-2'-deoxyuridine was counted in a gamma counter. A standard curve of porcine TGF-β was included in each assay and data were expressed as fmoles of TGF-β_1.

3.2.8 Purifying the Viral Particles

To purify the viral particles it was necessary to collect 800 ml of viral supernatant. Retroviral infected cells were seeded at $1.0x10^6$ in 40x150 mm plates with 20 ml of α MEM 10% FBS and incubated at 37°C for a week. The media was changed every 2-3

days. When the cells were subconfluent, the media was changed for a final time with 20 ml of αMEM 10% FBS and the supernatant was collected the next day in a 1 liter beaker and covered with saran wrap. The supernatant was stirred for half an hour after adding 50g NaCl at room temperature. Then the beaker was taken to the cold room and 360 ml of 40% PEG solution was added. The beaker was left overnight in the cold room. Next day the virus particles were pelleted by spinning the supernatant at 17,000 x g for half an hour in a 4°C centrifuge. The supernatant was discarded and the pellet was resuspended in 5 ml PBS and transfered to a small beaker. The solution was stirred overnight at 4°C.

The next day the concentrated virus solution was run on a density gradient comprised of potassium tartrate to isolate the viral particles. The gradient was prepared using two different concentrations of potassium tartrate that were poured into two columns. The more concentrated 35% potassium tartrate solution was poured into the first column, allowed to seep through an open valve into the second chamber and then the valve was shut. The less concentrated 20% potassium tartrate solution was poured into the second chamber. A pump connected to the two chambers was then turned on and the more concentrated solution mixed into the less concentrated potassium tartrate solution, making a final volume of 8 ml in an ultracentrafuge tube. The 5 ml of viral particles that had been dissolved in the PBS was added to the gradient in the ultracentrifuge tube. The density gradient was spun at 4°C at a speed of 30,000 x g for 6 hours. Once the ultracentrifuge stopped the density gradient was checked for a faint white circular band. Due to the uncertainty of where the viral particles were situated in the gradient, the whole gradient was fractionated. Each fraction was checked for the presence of viral particles

by infecting NIH 3T3 cells. Once the proper fractions containing the viral particles were identified the viral particles were quantitated by titration.

3.2.9 In vivo Instillation of Viral Particles

The p1521/TGF-β_1 vector induction is under the control of a mouse metallothionine promoter inducible by zinc. This suggests that for TGF-β_1 induction to occur adequate levels of zinc must be present in the rat lungs. For this reason we first determined how much zinc sulfate dissolved in the drinking water of the animals would by adequate to increase zinc levels in the blood and lungs. To determine this, a number of rats were given different amounts of zinc sulfate in their drinking water. Then their blood was sent to the Health Sciences Chemistry laboratory to quantitate zinc sulfate levels. In this manner the quantity of zinc sulfate in the drinking water required to elevate the serum zinc levels from normal baseline was determined.

Retroviral vectors are best assimilated into actively proliferating cells. We know from previous work that administration of bleomycin leads to alveolar epithelial cell damage, DNA synthesis and proliferation (Khalil et al, 1994). For this reason a smaller dose of bleomycin consisting of 0.1 units which was previously demonstrated to induce alveolar epithelial cell injury and proliferation but not fibrosis was used. Administration of 0.1 units of bleomycin also induces alveolar macrophage proliferation (personal communication with Dr. Khalil). Female Sprague-Dawley rats free of respiratory disease (assessed by Animal Care Facility U of M) were injected with 0.1 units of bleomycin +/- virus. The bleomycin was administered concomitantly with the viral particles. The rats

were anesthetized with 100 µl Ketaline/ 60 µl Rompum cocktail. Tracheostomy was performed and an intra tracheal injection of 0.1 units of bleomycin (200 µl) and virus (400 µl) which contained the LTGF-β was administered. Control rats only received 0.1 units of bleomycin and no viral particles.

The tracheal site of surgery was sutured and rats were allowed to recover until the time of sacrifice. The drinking water contained zinc sulfate (2.8g zinc/liter of water) to induce the metallothionine promoter to express the TGF-β gene. On the fifth day under Nembutal narcosis a thoractomy was done to expose the heart and lungs. The lungs were lavaged using normal saline and alveolar macrophages that were collected were first counted and then plated at a density of $2.5x10^5$ cells/ 60 mm tissue culture plates. The cells were allowed to adhere for 2 hours. The plates were then washed with αMEM and cultured in 0.2% BCP. The explanted alveolar macrophages were cultured in the presence and absence of 100 µM zinc sulfate to induce the metallothionine promoter for TGF-β_1 gene expression. Conditioned media was collected in the presence of protease inhibitors to prevent degradation of TGF-β_1 and the samples were stored in -80°C (Box 1-4) until detection and quantitation of TGF-β.

3.3.0 Active TGF-β_1 Construction

A retroviral vector carrying an active form of TGF-β_1 cDNA was not available to us. To construct this vector we digested a porcine TGF-β_1 cDNA out of the pPK9A plasmid and ligated the insert into the empty retroviral vector designated p1521 given to our lab by Dr. Mary Lou Cuttler. It was necessary to cut the insert out of the pPK9A

vector and re-ligate it into a new retroviral vector because retroviruses contain 5′ and 3′ LTR regions and a packaging signal that allows for packaging of the vector into an infectious particle and for cellular integration.

The pPK9A vector was grown large scale and then purified using the Qiagen Plasmid Kit (Qiagen Inc.) without modification in the protocol. Primers (5′ and 3′) to the TGF-β insert were designed using the oligomer program available to students in the Manitoba Institute of Cell Biology. Once the primer sequences were chosen they were ordered from Gibco BRL. The PCR reaction was prepared in a total of 100 µl using 100 pM of each primer and 20 ng of the pPK9A containing the TGF-β insert. A thermal cycler was programmed to amplify the insert for a total of 25 cycles. To ensure that the insert had been amplified by PCR, a 10 µl aliquot of the PCR product was run on a 0.7% agarose gel and electrophoresed for 1-2 hours. A photograph of the 1200 bp TGF-β fragment was taken for permanent record. The remaining 90 ul of the PCR product was purified using a gene clean Kit II (Gene Clean) without modification. To estimate the concentration of the insert, 1 µl of the PCR product was run on a 0.7 % agarose gel beside a Lambda Hind III marker of known concentration. A photograph was again taken for a permanent record.

In order to ligate the TGF-β insert into the retroviral vector designated p1521 it was necessary to change the 5′ and 3′ flanking sequences of the TGF-β insert from Bgl II to EcoRI sticky ends. Only the 5′ flanking sequence prior to the start codon of the insert was modified by a single base so that the insert could get ligated into the EcoRI site of a

TA vector (TA vector Kit). (ie. Original sequence CCC ATG was modified in the 39 mer to ACC ATG). A ligation reaction of 10 µl was prepared using 2 µl of the TA vector, 2 µl of the TGF-β insert, 1 µl of T4 DNA ligase , 1µl 10X Buffer and 4 µl of sterile water. The reaction was incubated overnight in a 4°C waterbath. Next day 1 µl of the ligation mixture was eletroporated in DH5α cells and then plated onto LB plates containing 60 µg/ml ampicillin Ten colonies were picked to do plasmid minipreps and each clone was restriction digested with EcoRI to check for the correct sized insert. Prior to ligating the TGF-β insert into the retroviral vector, it was sequenced to ensure that the reading frame had not changed. Once the sequence was verified the insert was cut out of the TA vector and ligated directly into the EcoRI site of the retroviral vector. The ligation mixture was again electroporated into DH5α cells and then plated onto LB plates containing 50 µg/ml Kanamycin. Colonies were picked and plasmid minipreps and restriction digests were carried out to identify the clones with the correct sized insert. The clones were sequenced and one was picked to grow up large scale. This clone was then used to package the retroviral vector into an infectious particle.

3.3.1 DNA Sequencing

The Porcine TGF-β_1 cDNA was sequenced using two different sequencing protocols, a T^7 sequencing kit or via an automatic sequencer. The T^7 sequencing Kit (Pharmacia Biotech) was used according to manufacturers instructions. Samples were labeled with ^{35}S and then run on a 6% acrylamide gel for 3-5 hours. The gel was soaked in a 10% acetic acid/ 10% methanol solution to fix the sequences onto the gel and then

the gel was dried onto a filter paper for 2 hours at 80°C. The sequence was exposed using film that was placed into a cassette for 2-3 days at room temperature.

A new automatic sequencer (ABI PRISIM 310) that requires a thermal cycler was also used to determine some sequences. This procedure employs the use of an ABI sequencing kit and a total reaction volume of 5 µl. In a 1.5 ml microfuge tube, 2 µl of terminator ready reaction mix, 125 ng of double stranded DNA, 2.5 picomole primer and enough sterile water was added to make a final volume of 5 µl. A thermal cycler was programmed to run the following conditions: 96°C for 10 sec, 40°C for 5 sec, 60°C for 4 minutes, for a total of 25 cycles. Then the amplified products were held at a 4°C hold temperature. The extension products were purified by ethanol precipitation and the DNA was pelleted by centrifugation at top speed for 15 minutes. The pellet was handed over to the technical staff responsible for the operation of the sequencing machine.

3.3.2 Correction of Cytosine Nucleotide Deletions

The porcine active TGF-β cDNA that was given to our lab was first sequenced prior to using it for retroviral vector construction. Upon sequencing the cDNA, we found a number of mutations within the first 30-40 nucleotides at the 5′ end of the gene.

Mutations in Sequence
ATG GCG CCT TCG GGG CTG CGG CTC TTG CCG TGC TGC TGC C

Correct Sequence

ATG CCG CCT TCG GGG CCT GGG CTC TTG CCG CTG CTG CTG CC

This caused a frame shift in the reading frame and lead to the translation of a nonfunctional protein. To correct the problem it was necessary to employ the technique of site directed mutagenesis and PCR technology.

A 39 base pair oligomer was made using the oligo-program on the student computer in the Department of Cell Biology at the Cancer Foundation.

ACC ATG CCG CCT TCG GGG CCT GGG CTC TTG CCG

This oligomer was purchased from Gibco BRL. It was necessary for the oligomer to be this long because the mutations spanned up to the 35th nucleotide at the 5$'$ end of the gene. A PCR reaction was prepared for site directed mutagenesis in the following manner: The entire reaction mix was 100 µl consisting of, 10 µl 10X PCR buffer, 10 µl dNTP's (2 mM), 5 µl of the 5$'$ primer (20 pM/µl), 5 µl of the 3$'$ primer (20pM/µl), 2µl of template DNA (20 ng of pPK9A), 0.5 µl of Taq polymerase, and 67.5 µl of sterile distilled water. The thermal cycler was programmed so it would run 96° C for 10 sec, 65° C for 5 sec, 60° C for 4 minutes and a 4° C hold temperature for a total of 25 cycles. After the reaction was completed, the entire reaction mix was purified using "gene clean" (Gibco BRL) and then 10 µl of the reaction was run on a 0.7% agarose gel to see if the correct band was there. Once the correct size was confirmed, the porcine TGF-β cDNA

was ligated into the TA vector and incubated at 4°C overnight. One microliter of the ligation mix was electropated in DH5 α cells as described elsewhere. An aliquot of 200 µl of culture was plated out on ampicillin (100 µg/ml & 20 µl Xgal) plates overnight. Next, 10 colonies were picked and suspended in 5 ml of LB broth with ampicillin and incubated overnight in a 37°C shaker. Five clones were picked and sequenced as described elsewhere. The clone with the correct sequence was cut out with Eco RI and the insert was purified using "gene clean". The empty retroviral vector designated p1521 was cut open at the EcoRI site and dephosphorylated with calf intestinal phosphatase (Pharmacia). This was necessary so that the vector does not re-anneal with itself during the ligation reaction. Once the vector was dephosphorylated, it was also gene cleaned to purify it. Before setting up the ligation reaction 2 µl of the insert and 2 µl of the vector were run on a 0.7% agarose gel to estimate the concentrations. The ligation reaction was prepared using 2 µl of vector, 2 µl insert, 1 µl of 10X PCR buffer, 1 µl T4 DNA ligase (Pharmacia) and 4 µl of sterile water. The reaction was incubated overnight at 14°C and then an aliquot was transformed in DH5α cells to select for the retroviral clones that had taken up the porcine TGF-β cDNA. The clones were randomly picked and sequenced to check if the mutation had disappeared. A retroviral clone with the correct sequence was found and packaged into infectious particle as described elsewhere.

3.3.3 In vitro Transcription/Translation

Since we were unable to detect any protein expression with the CCL 64 bioassay, it was necessary to do *in vitro* transcription/ translation on the CMV controlled active TGF-

β_1 plasmid to confirm whether the TGF-β cDNA was being translated into protein. A postdoc in Dr. Greenberg's lab (MICB) conducted this test. He used a Rabbit Reticulocyte lysate kit (Promega). In a 1.5 ml microfuge tube he prepared a 50 µl reaction mixture that contained 1 µg of template, the lysate, and all the amino acids except the methionine which was added as ^{35}S labeled methionine. Then T^7 polymerase and Rnase inhibitors were added and the reaction was incubated at 30°C for 1 ½ hours. At that time sample buffer was added (glycerol 10%, 2-β mercaptoethanol 0.2%, SDS 3%, 0.5 M Tris HCl final concentration 0.0625M) to ensure that the contents of the reaction mixture sink into the well once loaded into the gel. The reaction mixture was run on a SDS-page mini gel beside a marker for 1 hour at 150 volts. The gel was then fixed in 10% acetic acid/ 25% isopropanol for ½ an hour. The signal of ^{35}S was amplified by soaking the gel in amplifying buffer (Amersham) for ½ hour. The gel was then dried at 80°C for 1 hour and then exposed. The *in vitro* transcription/translation was carried under reducing (addition of mercaptoethanol) and non- reducing conditions (absence of mercaptoethanol).

3.3.4 ELISA

The ELISA kit was purchased from R&D systems. This assay is a quantitative sandwich enzyme immunoassay. The procedure in the kit was followed without any modification. The principal behind the assay is that TGF-β soluble receptor Type II binds TGF-β_1 that has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TGF-β_1 present is bound by the immobilized receptor.

The unbound substances are washed away, and enzyme linked antibody specific to TGF-β_1 is added to the wells to sandwich the TGF-β_1. A substrate solution is added to the wells for a color development. The intensity of the color is measured by taking absorbency readings at 450 nm.

4. Results

4.1. Quantitating In Vitro Gene Expression

Once the retroviral vector p1525 was packaged into infectious particle, it was necessary to infect a cell line and determine if the metallothionine promoter could be induced to express the LTGF-β_1 cDNA. NIH 3T3 cells were infected with virus carrying the LTGF-β_1 cDNA and growth selected with geneticin for a period of two weeks so the entire population was infected with at least one viral particle. The cells were cultured in the absence or presence of zinc sulfate (100 μM). From the infected cells conditioned media was collected at 24 hour (Table 6) and 48 hour (Table 7). A CCL 64 bioassay was done to determine the amount of TGF-β secreted from the cells under reducing and non-reducing conditions.

Table 5:

LTGF-β_1 Secreted by NIH 3T3 Cells Stably Infected with Virus Containing cDNA for LTGF-β_1

	Neutral Conditioned Media fmoles/ 24 hours 2.0×10^5 Cells		Acidified Conditioned Media fmoles/ 24 hours 2.0×10^5 Cells	
	Uninfected Cells	**Infected Cells**	**Uninfected Cells**	**Infected Cells**
	0.0	0.0	6.78	18.80
	0.0	0.0	8.45	19.00

Conditioned media obtained after 24 hours does not show any real difference between cells growing in the absence or presence of zinc sulfate. It is possible that gene expression by transfected cells requires more than 24 hours to detect the induction of LTGF-β_1.

Table 6:

LTGF-β_1 Secreted by NIH 3T3 Cells Stably Infected with Virus Containing cDNA for LTGF-β_1

Zinc Sulfate µM	Neutral Conditioned Media fmoles/48 hour		Acidified Conditioned Media fmoles/ 48 hours 2.0×10^5 Cells	
	Uninfected Cells	**Infected Cells**	**Uninfected Cells**	**Infected Cells**
	0.0	0.0	0.0	37.0
	0.0	0.0	38.6	186.0

Conditioned media obtained 48 hours after culture showed a considerable increase in the amount of LTGF-β_1 secreted. It is of interest that when transfection of cells was done the amount of LTGF-β_1 protein that was produced prior to increasing the viral particles by the ping pong method was 106 fmoles compared to 186 fmoles after ping pong amplification of the virus particles. This suggests that the copy number of the gene did increase and resulted in an increase in TGF-β_1 synthesis and release.

4.2.2. Quantitating In Vivo Gene Expression

Female Sprague-Dawley rats were intra-tracheally injected with 0.1 units of bleomycin in the absence or presence of virus vector. To induce LTGF-β gene

expression via the metallothionine promoter. their drinking water contained zinc sulfate (2.8g zinc sulfate/liter water). The rats were sacrificed 5 days later and their alveolar macrophages were lavaged from their lungs and cultured in serum free media. Conditioned media was collected after 24 hour. 48 hour and 7days in culture and assayed for amount of LTGF-β_1 present.

Table 7:

Amount of LTGF-β_1 Secreted by Rat Alveolar Macrophages in 24 Hours

Treatment of Rats	TGF-β_1 in conditioned media containing 100 μM Zinc Sulfate fmoles/24 hours	
	Neutral	Acidified
Intratracheal delivery of 0.1 unit Bleomycin No viral particles administered	4.6	8.75
Intratracheal delivery of 0.1 unit Bleomycin 400 μl of viral supernatant (media) ($1.0x10^5$ CFU/ml)	6.6	0.0

Table 8:

Amount of LTGF-β_1 Secreted from Rat Alveolar Macrophages in 48 Hours

Treatment of Rats	TGF-β_1 in conditioned media containing 100 µM Zinc Sulfate fmoles /48 hours	
	Neutral	Acidified
Intratracheal delivery of 0.1 unit Bleomycin No viral particles administered	1.53	3.45
Intratracheal delivery of 0.1 unit Bleomycin 400 µl of viral supernatant (media) ($1.0x10^5$ CFU/ml)	1.12	9.15

Conditioned media obtained from cultured macrophages of the animal that received bleomycin and virus particles showed the most amount of TGF-β present. The macrophages that served as a control showed considerably less TGF-β in the conditioned media. The CCL 64 Bioassay was also performed on the same number of alveolar macrophages plated in the absence of any zinc treatment. These samples had no increase in LTGF-β_1 present.

Table 9:

Amount of LTGF-β_1 Secreted by Rat Alveolar Macrophages in 7 Day s

Treatment of Rats	TGF-β_1 in conditioned media containing 100 μM Zinc Sulfate fmoles/7days	
	Neutral	Acidified
Intratracheal delivery of 0.1 unit Bleomycin No viral particles administered	3.40	0.0
Intratracheal delivery of 0.1 unit Bleomycin 400 μl of viral supernatant (media) ($1.0x10^5$ CFU/ml)	2.70	16.5

There was an increased amount of LTGF-β_1 present in the 7 day conditioned media compared to the other time points. Another group of rats with the following treatments, (0.1 units of bleomycin, 0.1 units of bleomycin and 400 μl of viral supernatant, 400 μl of αMEM media the viral supernatant was collected in) were sacrificed after 5 days and their lungs were sectioned and stained with hemotoxylin and eosin. Although after *in vivo* administration of retroviral vector 1525 there was significant alveolar macrophage induction of secretion of LTGF-β_1, there was no evidence of inflammation confirming that for TGF-β to be effective it must be in its biologically active form.

4.2.3. Quantiating Porcine Active TGF-β_1 Expression

A CCL 64 bioassay was performed on the media collected from NIH 3T3 cells that were stably transfected with the p1521/ TGF-β_1 and maintained in culture for 24 hours and 48 hours.

Table 10:

Amount of TGF-β Secreted from NIH 3T3 Cells Stably Infected with Porcine Mutagenized TGF-β cDNA

Zinc Sulfate µM	Neutral Conditioned Media fmoles/24 hours 2.0×10^5 Cells		Neutral Conditioned Media fmoles/48 hours 2.0×10^5 Cells	
	Uninfected Cells	**Infected Cells**	**Uninfected Cells**	**Infected Cells**
	0.42	0.88	0.0	5.51
	0.29	0.43	0.12	3.41

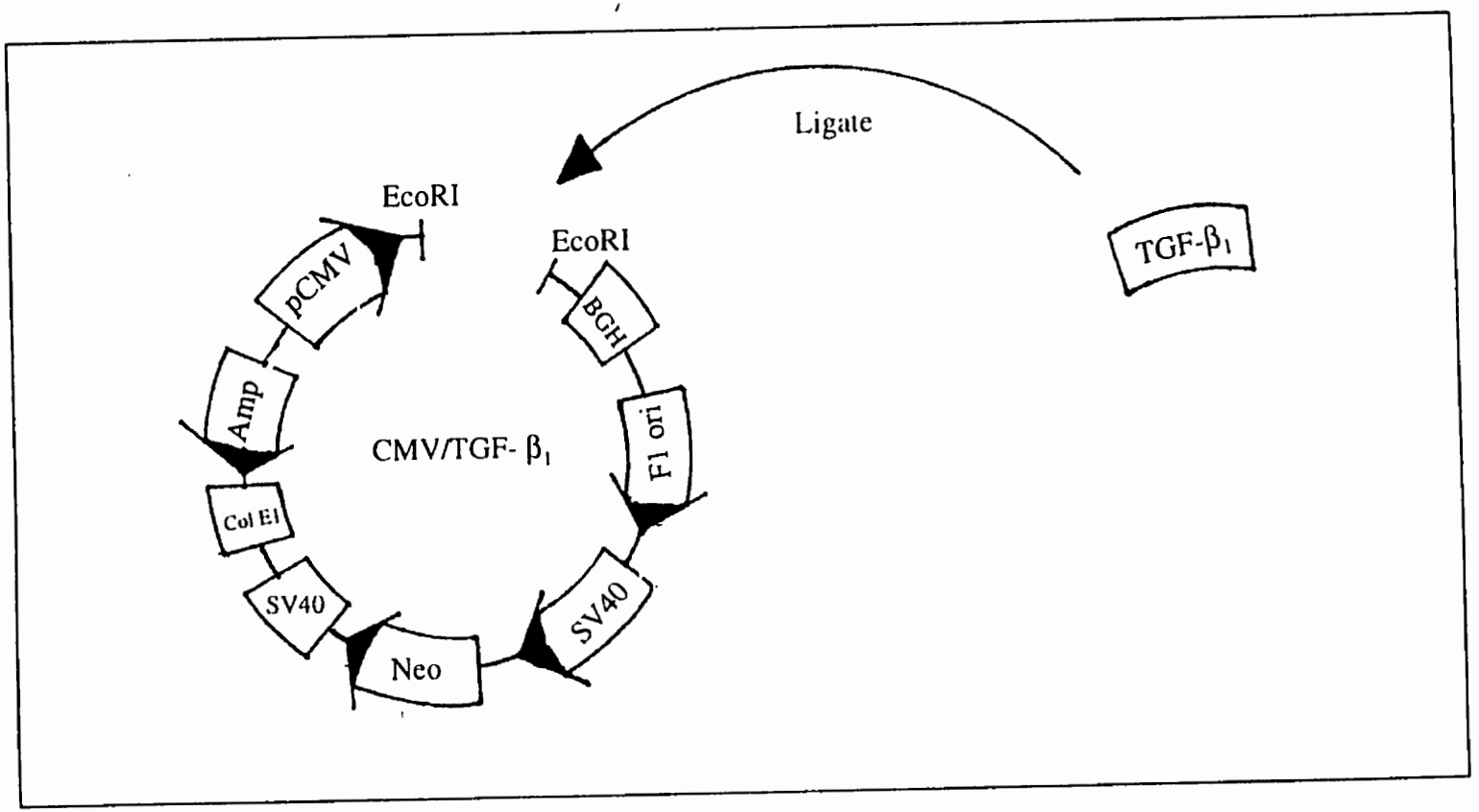
The amount of TGF-β_1 present in neutral conditioned media is not very high and raised concerns that other proteins in the conditioned media in addition to the TGF-β_1 may be affecting the CCL 64 epithelial cells of the assay.

4.2.4. Construction of PC DNA 3.1

Due to a low level of gene expression from retroviral vector p1525/TGF-β_1 carrying the bioactive form of TGF-β_1, a new construct was made with a CMV promoter controlling gene expression. The porcine active TGF-β_1 cDNA was restriction digested out of the retroviral vector p1521/TGF-β_1 and ligated into a CMV controlled pcDNA 3.1 plasmid (Figure 7). Individual clones were isolated and growth selected with geneticin. A CCL 64 bioassay was performed on each of the following cell lines: 1) NIH 3T3 , 10T1/2 and A549.

There was no detectable TGF-β in conditioned media when measured using the CCL 64 bioassay. In the past we had found that when a sample contains very large quantities of TGF-β the cells of the CCL 64 bioassay are either not growth inhibited or are induced to proliferate. However dilution of the test sample can then result in demonstrating the anti-proliferative effects of TGF-β on this assay. A possible reason for not detecting any TGF-β_1 could be that there may have been a very high concentration of TGF-β_1 present in the conditioned media that impaired the assay. For this reason the conditioned media was diluted 1/10 and 1/100 and the CCL 64 assay done. However, no detectable TGF-β was present. It was also possible that the quantity of TGF-β secreted was very low due to the small number of cells used for transfection. In the next experiments we increased the number of cells that were plated prior to collecting conditioned media. The increase in the number of infected cells did not result in detectable TGF-β_1.

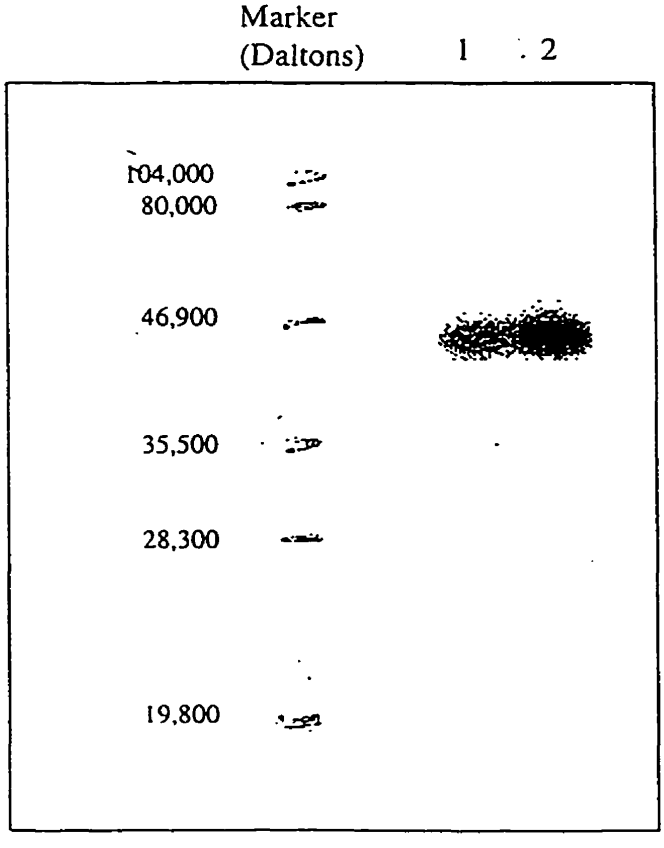
Figure 7: Map of CMV PcDNA 3.1 Carrying Bioactive TGF-β_1



4.2.5. In Vitro Transcription/Translation

Since the protein expression as detected by the CCL 64 bioassay did not demonstrate the presence of TGF-β, we next determined if the gene for TGF-β_1 was being transcribed and translated off the CMV promoter using an *in vitro* transcription/translation kit and SDS page gel to detect the size of protein produced. According to the literature, monomeric porcine TGF-β_1 protein is 45 kDa in size (Sha et al, 1989). The TNT Rabbit Reticulocyte lysate kit (Promega Inc.) was used for *in vitro* transcription/translation under reducing and non-reducing conditions. Upon exposure of the film, a 45 kDa sized band was detected.

Figure 8: In vitro Transcription/Translation



Lane 1: non-reducing conditions
Lane 2: reducing conditions

This confirmed that the monomeric protein that is being transcribed and translated is porcine TGF-β_1 and therefore should have been present in the conditioned media. Since it was possible that a substance in the conditioned media could be impairing the detection of TGF-β in the CCL 64 bioassay, we used an ELISA kit to carry out an immunoassay specifically detecting TGF-β_1. We were unable to detect TGF-β_1 by the ELISA. None of the samples showed values that appeared on the standard curve. The amount of TGF-β_1 in the samples was far below the lowest dilution of the standard curve which detects 10 fmoles of TGF-β_1.

Discussion

Idiopathic pulmonary fibrosis is a relentless and lethal fibrotic lung disease. As it progresses, the lung becomes less and less able to transfer oxygen from air to blood in a normal fashion (Wolff and Crystal, 1997). It is characterized by the recruitment of inflammatory cells that are activated to release proinflammatory and fibrogenic cytokines at the site of tissue injury. Of these inflammatory cells, the alveolar macrophages have been shown not only to increase in number during pulmonary injury (Chandler et al, 1983), but they have also been shown to produce a number of fibroblast regulating cytokines such as IL-1 (Jordana et al, 1988), PDGF (Martinet et al, 1987), TNF-α (Bachwich et al, 1986), and TGF-β (Khalil et al, 1989). Of the cytokines produced TGF-β_1 is the most potent regulator of events leading to lung fibrosis (Coker and Laurent, 1997). It can induce transcription of other growth factors such as IL-1, PDGF, bFGF, TNF-α and TGF-β itself (Wahl et al, 1987; McCartney et al, 1988). TGF-β_1 is a multifunctional cytokine that is not only a mitogen for immature fibroblasts (Hill et al, 1986) but also a chemoattractant for fibroblasts (Postlethwaite et al, 1987) and induces connective tissue synthesis which leads to fibrosis (Ignotz and Massague, 1986). A study done by Broekelmann et al, (1991) demonstrated that in biopsy material taken from IPF lungs, TGF-β was localized by immunohistochemistry at the exact sites where cells in that area were actively making mRNA for collagen. Furthermore, in an immunohistochemical study Khalil et al, (1996) demonstrated that TGF-β_1 is aberrantly present in alveolar macrophages in early lesions from patients with IPF. However, in advanced pulmonary lesions from the same patients TGF-β_1 was present not only in alveolar macrophages but also in alveolar epithelial cells irrespective of the etiology.

Normally alveolar epithelial cells do not produce TGF-β_1 (Khalil et al, 1996). Based on this information we sought to determine if aberrant expression of TGF-β_1 by alveolar epithelial cells was important in regulating inflammation and fibrosis in the lung. We hypothesized that if we overexpress TGF-β_1 in epithelial cells this will lead to increased localized inflammatory changes and connective tissue synthesis. To chronically overexpress TGF-β_1 *in vivo* we developed three retroviral vectors. The vector, p1525 was a gift from Dr. Mary Lou Cuttler (NIH) and contained the cDNA for LTGF-β_1, a metal induced metallothionine promoter, a neomycin drug marker, and a packaging signal. This retroviral vector was packaged into infectious particles using a ψ_2 packaging cell line. Ecotropic virus produced from each clone was then collected in vials and frozen at -80°C. The viral titer from each clone was determined in CFU/ml. Prior to doing any *in vivo* work it was necessary to first test infectivity of the virus *in vitro*. By infecting a cell line *in vitro* we could confirm whether the MT-1 promoter controlling the expression of the LTGF-β_1 cDNA was intact and responsive to zinc sulfate induction. NIH 3T3 cells were infected with the virus carrying the LTGF-β_1 cDNA and growth selected in geneticin for approximately two weeks to ensure that the entire population of cells were infected. This cell line was chosen because it was easily accessible at MICB (Manitoba Institute of Cell Biology) and several laboratories are experienced in using this cell line for their own work. To quantitate protein expression a CCL 64 bioassay was performed on infected and non-infected NIH 3T3 cells cultured in the presence or absence of zinc sulfate, and the amount of protein that was secreted by these cells in conditioned media was quantitated. However, the results showed that level of protein secreted was almost the same as the control conditions. My committee members

suggested that I should increase the titer of the virus using a method called "ping pong" amplification (Kozak and Kabat, 1990) prior to testing the virus *in vivo*. This method is a successful way of increasing the number of viruses that can infect a single cell. Hence there is more expression of the gene when the promoter is induced. Alternative methods that could have been applied to increase the titer are described by Vanin et al (1997) in which they develop a high-titer retroviral producer cell line by using Cre-mediated recombination and Lynch and Miller (1991), who describe the production of high-titer helper virus-free retroviral vectors by co-cultivation of packaging cells with different host ranges. Both these methods required making changes to the retroviral vector backbone prior to using the protocol and therefore the "ping pong" amplification method was applied. Following "ping pong" amplification, individual ψ_2 clones were isolated and their respective titers were determined in CFU/ml. NIH 3T3 cells were growth selected for approximately two weeks and a CCL 64 bioassay was performed on the infected cells. This time the amount of LTGF-β_1 was almost twice the amount of protein detected prior to ping pong amplification suggesting that the number of the viruses infecting a single cell most probably increased.

The viral particles obtained from the packaging cell line contained 10% FBS that was used to culture the packaging cell line. Viral particles in FBS given *in vivo* to rats would be expected to elicit an independent inflammatory and immunological response. For that reason the next step was to purify the viral particles from the media supernatant and to suspend them in a biological inert vehicle such as normal saline. For this purpose approximately ten liters of viral supernatant was collected, centrifuged, the viral particles

pelleted, resuspended and run on a density gradient. The viral band was not visible and could have been present in many regions of the density gradient. The entire gradient was fractioned and 1µl of each fraction was used to infect NIH 3T3 cells. The cells that survived after growth selection with geneticin indicated which fraction contained the viral particles. That fraction was next titered to determine CFU/ml. Surprisingly, the CFU/ml did not increase and as a result the volume of purified virus was much lower than imagined. Even so, this purified viral concentrate suspended in normal saline was used for *in vivo* instillation. However, prior to that it was first necessary to determine the amount of zinc sulphate that should be dissolved into the drinking water of female Sprague Dawley rats to ensure the MT-1 promoter induction. A dose response with zinc sulfate treated water (5.6 g/liter of water, 2.8 g/liter of water and 1.2 g/liter of water) was given to 3 rats, as well as a control rat drinking plain water. The rats received this treatment for 7 days after which they were sacrificed and their blood tested for zinc sulfate content. The rat drinking 2.8 g/ liter of water appeared to be the best choice out of the three rats. The rat drinking 5.6 g/ liter had lost too much weight over the 7 day period and had developed bloody lesions on the eyelids. The rat drinking 1.2 g/ liter had a lower zinc sulfate level in the blood test compared to the one drinking 2.8 g/ liter.

Rats were injected with virus intratracheally either concomitantly with bleomycin (0.1 units) or normal saline. The bleomycin was used at a very low dosage (0.1 units) enough to cause minor local injury and cells in the region to proliferate. The rats were on zinc sulphate water until the time they were sacrificed on day five. Their lung macrophages were lavaged out of the lungs and plated into tissue culture plates. The

reason why the macrophages were lavaged out of the lung was because they are easier to remove than epithelial cells and secondly, it was possible that the alveolar macrophages were infected as well. By doing a CCL 64 bioassay it was determined that LTGF-β_1 was elevated in the seven day conditioned media from rats receiving bleomycin and virus compared to little or none in the 24 hr and 48 hr conditioned media from the same treatment animals. This shows that macrophages needed to be cultured in the presence of zinc sulphate for a few days before the gene for LTGF-β_1 could be expressed.

Since the gene for LTGF-β_1 could be expressed using the retroviral vector, it seemed appropriate to construct a new viral vector that contains the active TGF-β_1 cDNA. For this purpose plasmid pPK9A was used because it contains the mutegenized porcine LTGF-β_1 cDNA. The mutagenesis is a result of converting Cys 223 and Cys 225 amino acid residues to serines, resulting in mature TGF-β_1 that no longer has a non-covalent associated LAP. The TGF-β_1 cDNA was restriction digested out of the pPK9A plasmid and ligated into the empty retroviral vector provided by Dr. Mary Lou Cuttler. This unfortunately resulted in problems because upon sequencing the new retroviral vector carrying the mutagenized porcine TGF-β_1 cDNA, I discovered a series of cytosine nucleotide mutations in the 5′end of the gene, resulting in a sequence that translates into a non-functional protein. To correct the mutations, a 39 nucleotide long oligomer was designed and by using PCR technology the mutation in the 5′ end of the gene was corrected. The gene was sequenced and the correct sequence was confirmed.

Following this, it seemed appropriate to package the newly constructed retroviral vector using ping pong amplification. In the same manner as described earlier the clone with the highest titer was isolated. The virus was infected into NIH 3T3 cells and after growth selection protein was quantitated using the CCL 64 bioassay. Surprisingly, no protein was detected with the assay. The assay was repeated a number of times but no evidence of TGF-β was found. We felt that it was possible the promoter was not strong enough for gene expression so the corrected TGF-β_1 cDNA was restriction digested and ligated into a PC DNA 3.1 plasmid carrying a very strong CMV promoter. The newly constructed plasmid was sequenced and confirmed to be correct. Another way to confirm the gene was not defective was by doing *in vitro* transcription/translation. This was done by using a Rabbit Reticulocyte lysate kit. The protein was labeled with ^{35}S methionine. The resulting protein when run on a SDS page gel was 45 kDa in size, which is the exact size of monomeric TGF-β_1. Since a monemeric TGF-β_1 was present, we were confident that the expression vector would work. Three cell lines, NIH 3T3, 10T1/2, and A549 were infected and growth selected in geneticin. CCL 64 bioassays were performed on each of the cell lines and no detectable TGF-β was present. Since it was possible that a substance in the conditioned media could be impairing the detection of TGF-β in the CCL 64 bioassay, we used an ELISA kit to carry out an immunoassay specifically detecting TGF-β_1. We were unable to detect TGF-β by the ELISA. My supervisor suggested that the number of cells plated onto the tissue culture plates prior to collecting conditioned media might have been too low, and if there was any protein being secreted it is not enough to be detected.

A final attempt was made to see if the gene expression from the three cell lines could be detected *in vitro*. Conditioned media was collected 24 hours, 48 hours, and 72 hours from 6 million cells instead of $2.5x10^5$ cells. An ELISA kit was purchased again, and the TGF-β_1 immunoassay was carried out. All the clones were tested, however the results showed that there was still no detectable TGF-β_1 present in the samples. None of the samples showed values that appeared on the standard curve. The amount of TGF-β_1 in the samples was far below the lowest dilution of the standard which detects 10 fmoles of TGF-β_1. From this we concluded that it is possible that the protein is not being properly processed inside the cell and therefore is not secreted outside the cell. Sha et al (1989), have demonstrated that oligosaccaride additions to pre-pro-TGF-β_1 are important for secetory exit of TGF-β from the cell, but play no role in the specific proteolysis of the pre-pro-TGF-β. It has also been demonstrated that mutagenesis of the glycosylation sites in the TGF-β_1 pro region result in undetectable amounts of TGF-β protein secretion (Brunner et al, 1992). Therefore it is possible that the cDNA for porcine TGF-β_1 has altered glycosylation sites leading to lack of secretion of TGF-β in all of the samples. To confirm this speculation infected and control cells can be used for total protein extraction and western analysis to indirectly quantitate the intracellular protein levels. If we find an increase in intracellular content of TGF-β_1 future studies to determine the location of intracellular TGF-β_1 could be done by quantitating TGF-β_1 in subcellular compartments of infected cells. Another way to resolve this issue would be to go back to the original retroviral vector p1525 (gift form Dr. Cutler) that carries the LTGF-β cDNA and mutagenize the cysteine residues at positions 223 and 225 using PCR technology. This seems plausible because I have already shown that *in vitro* the retroviral vector expresses

LTGF-β in great abundance and the promoter is intact and inducible in the presence of zinc sulfate. I would also insert a lac Z gene in the retroviral backbone so that the virus can be localized in the tissue by using in situ hybridization.

Cytokine gene transfer to the lung epithelium has been successfully attempted by many researchers. McCray et al (1995), used an adenovirus vector to transfer a gene to the fetal pulmonary epithelia *in vitro* and *in vivo*. Korfhangen et al (1994), developed transgenic mice that expressed human transforming growth factor alpha in respiratory epithelial cells and Mastrangeli et al (1993), describe targeting genes to the airway epithelium using *in vivo* recombinant adenovirus-mediated gene transfer. The use of adenoviral vectors is considered a favorable vehicle to overexpress cytokine genes transiently, such that their effect is like that seen during injury and repair (Gauldie et al, 1996). Dr. Jack Gauldie's group at McMaster University has done much research in the field of pulmonary fibrosis. They overexpressed TGF-β_1 in the lung airway epithelium using a replication deficient adenoviral vector carrying a mutagenized porcine cDNA for TGF-β_1 (Sime et al, 1997). They were able to show that up to 72.2 ng/ml of TGF-β_1 was recovered in BAL fluids of Sprague Dawley rats on the seventh day compared to 470 pg/ml in control animals (Sime et al, 1997). Quantitative measurement of collagen content was analyzed by hydroxyproline uptake. There was an increase in mean hydroxyproline in rats from day 14 (Sime et al, 1997). These findings published by Dr. Gauldie's group support my original hypothesis, stating that overexpression of TGF-β_1 by epithelial cells is important in inflammation and fibrotic changes seen in the lung.

This new and versatile model of pulmonary fibrosis can be used to conduct future studies concentrating on the pathological mechanisms of pulmonary injury.

Summary

1. Retroviral vectors were packaged into ψ_2 cells and viral titers were determined in CFU/ml.

2. Infectivity and gene expression was tested by infecting NIH 3T3 cells.

3. Amount of protein produced was quantitated using the CCL 64 bioassay.

4. Retroviral vector that contains the porcine Active TGF-β cDNA was constructed.

5. The vector was packaged, titered, and gene expression was quantitated *in vitro*.

References

Adams D.H., Hathaway M, Shaw J, Burnett D, Elias E, Strain A.J., (1991): Transforming growth factor-β induces human T lymphocyte migration in vitro. J. Immunology.147:609-612.

Adamson I, (1984): Drug-Induced Pulmonary Fibrosis. Environmental Health Perspectives. 55:25-36.

Albright J, Oppenheim J, (1991): Contributions of basic immunology to human health. FASEB J. 5:265-270.

Alevizopoulos A, Mermod N, (1997): Transforming growth factor-β: the breaking open of a black box. BioEssays. 19:581-591.

Altman D.J., Schneider S.L., Thompson D.A., Cheng H.L., Thomasi T.B., (1990): A TGF-β-2 like immunosuppressive factor in amniotic fluid and localization of TGF-β2 mRNA in the pregnant uterus. J. Exp. Med. 172(5):1391-401.

Attisano L, Wrana J, Casillas F, Massague J, (1994): TGF-β receptors and actions. Biochimica et Biophysica Acta 1222:71-80.

Bachwich P.R., Lynch J.P., Larrick J, Spegloe M, Kunkel S.L., (1986):Tumor necrosis factor stimulates interleukin-1 and prostaglandin E2 production in resting macrophages. Am. J. Pathol. 125:421-425.

Benn S.I., Whitsitt J.S., Broadley K.N., Nanney L.B., Perkins D, He L, Patel M, Morgan J, Swain W.F., Davidson J.M., (1996): Particle mediated gene transfer with transforming growth factor beta1 cDNA's enhances wound repair in rat skin. 98:2894-2902.

Bestwick R.K., Kozak S.L., Kabat D, (1988): Overcoming interference to retroviral superinfection results in amplified expression and transmission of cloned genes. Proc. Natl. Acad. Sci. USA. 85:5404-5408.

Border W.A., Ruoslahti E, (1992): Transforming growth factor -β in disease: the dark side of tissue repair. J Clin Invest. 90:1-7

Border W.A., Noble N.A., (1994): Transforming Growth Factor β In Tissue Fibrosis. The New England Journal of Medicine. 331:1286-1292.

Bottinger E.P., Valentina M.F., Tsang M.L., Weatherbee J.A., Kopp J.B., Qian S.W., Sporn M.B., (1996): The recombinant pro-region of transforming growth factor β1 (latency-associated peptide) inhibits active transforming growth factor β1 in transgenic mice. Proc. Natl. Acad. Sci. USA. 93:5877-5882.

Boyd F.T., and Massague, J.(1989): TGF-β inhibition of epithelial cell proliferation linked to the expression of a 53 kDa membrane receptor. J. Biol. Chem. 264(4):2272-2278.

Brandes M.E., Mai U.E.H., Ohura K, Wahl S.M., (1991): Human meutrophils express type I TGF-β receptors and chemotax to TGF-β. J. Immunology. 147:1600-1606.

Breen E, Shull S, Burne S, Absher M, Kelley J, Phan S, Cutroneo K, (1992): Bleomycin Regulation of Transforming Growth Factor-β mRNA in Rat Lung Fibroblasts. Am. J. Respir. Cell Mol. Biol. 6:146-152.

Broadly K.N., Davidson J.M., (1991): Transfection of wounds in vivo with transforming growth factor beta1 accelerates healing. J. Cell. Biochem. 15F:192.

Brokelemann T.J., Limper A.H., Colby T.V., McDonald J.A., (1991): Transforming growth factor beta1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc. Natl. Acad. Sci. USA. 88:6642-6646.

Brunner A.M., Lioubin M.N., Marqardt H, Malacko A.R., Wang W.C., Shapira R.A., Neubauer M, Cook J, Madisen L, Purchio A.F., (1992): Site-directed mutagenesis of glycosylation sites in the TGF-β1 and TGF-β2 precursors and of cysteine residues within mature TGF-β1:effects on secretion and bioactivity. Mol. Endo. 6(10):1691-700.

Castilla A, Prieto J, Fausto N, (1991): Transforming growth factors β1 and α in chronic liver disease: effects of interferon alfa therapy. N. Engl. J. Med.324:933-40.

Chandler D.B., Hyde D.M., Giri S.N., (1983): Morphometric estimates of infiltrative cellular changes during the development of bleomycin-induced pulmonary fibrosis in hamsters. Am. J. Pathol. 112(2):170-7.

Chantry D, Turner M, Abney E, Feldmann M, (1989): Modulation of cytokine production by transfroming growth factor-β. J. Immunol.142:4295-4300.

Chaudhury A. and D'Amore,(1991): Endothelial cell regulation by transforming growth factor beta. J.Cell. Biochem. 47:224-229.

Cheng L, Siegelohoffer P.R., Yang N.S., (1993): In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. Proc. Natl. Acad. Sci. USA. 90:4455-9.

Coker R.K., Laurent G.J.,(1997): Anti-cytokine approaches in pulmonary fibrosis: bringing factors into focus. Thorax. 52:294-296.

Connor T.B., Roberts A.B., Sporn M.B., Danielpour D, Dart L.L., Michels R.G., Bustros S, Enger C, Glaser B.M.,(1989): Correlation of fibrosis and TGF-β type 2 level in the eye. J. Clin. Invest. 83:1661-1666.

Coultas D.B., Zumwalt R.E., Black W.C., Sobonya R.E., (1994): The epidemiology of interstitial lung diseases. Am. J. Respir. Crit. Care Med. 150(4):967-72.

Crapo J.D., Barry B.E., Gehr P, Bachofen M, Weibel E.R., (1982):Cell number and cell characteristics of the normal human lung. Am. Rev. Respir. Dis. 126:332-7.

Crystal R, Bitterman P, Rennard S, Hance A, Keogh B, (1984): Interstitial Lung Diseases of Unknown Cause. New England Journal of Medicine. 310:154-163.

Curiel D, Pilewski J, Albelda S, (1996): Gene Therapy Approaches for Inherited and Acquired Diseases. Am. J. Respir. Cell Mol. Biol.14:1-18.

Czaja M.J.,Weiner F.R., Flanders K.C., (1989): In vitro and in vivo association of transforming growth factor-β_1 with hepatic fibrosis. J. Cell Biol. 108:2477-82.

Danos O, Mulligan R.C., (1988): Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc. Natl. Acad. Sci. USA. 17:189B-94B.

Ellingsworth L.R., Nakayama D, Segarini P, Dasch J, Carrillo P, Waegell W, (1988): Transforming growth factor-βs are epuipotent growth inhibitors of interleukin-1 induced thymocyte proliferation. Cell. Immunol.114:41-54.

Fontana A. Constam D.B., Frei K, Malipiero U, Pfister H.W., (1992): Modulation of the Immune Response by Transforming Growth Factor Beta. Int Arch Allergy Immunol. 99:1-7.

Gauldie J, Graham F, Xing Z, Braciak T, Foley R, Sime P, (1996): Adenoviral vector mediated cytokine gene transfer to lung tissue. Ann. N.Y. Acad. Sci. 796:235-244.

Hance A.J., Crystal R.G., (1975): The Connective Tissue of Lung. American Review of Respiratory Disease.112:657-711.

Hill D.J., Strain A.J., Elesturo S.F., Swenne S, Milner R.D.G., (1986): Bifunctional action of TGF-β on DNA synthesis in early passage human fetal fibroblasts. J. Cell Physiology. 128:322-328.

Holian A, Scheule R.K., (1990): Alveolar Macrophage Biology. Hospital Practice. 53-62.

Hunninghake G.W., Gadek J.E., Szapiel S.V., Strumf I.J., Kawanami O, Ferrans V.J., Keogh B.A., Crystal R.G.,(1980): The human alveolar macrophages. Methods Cell Biol. 21A:95-105.

Ignotz R.A., and Joan Massague,(1986): Transfroming growth factor beta stimulates the expression of fibronectin and collagen and their incorporation into the ECM. J.Biol, Chem. 261:4337-4345.

Ignotz R.A., Heino J, Massague J. (1989): Regulation of cell adhesion receptors by TGF-β. J. Biol. Chem. 264:389-392.

Ingrassia T.S., RYU J.H., (1991): Oxygen-Exacerbated Bleomycin Pulmonary Toxicity. Mayo Clin. Proc. 66:173-178.

Jetten A, Shirley J.E., Stoner G.(1986): Regulation of proliferation and differentiation of respiratory tract epithelial cells by TGF-beta. Exp. Cell. Res. 167:539-549.

Jin Y, Cox D.A., Knecht R, Raschodrof F, Cerletti N, (1991): Separation, purification and sequence identification of TGF-β_1 and TGF-β_2 from bovine milk. J. Protein Chemistry. 10(5):565-75.

Jordana M, Richards C, Irving L.B., Gauldie J, (1988): Spontaneous in vitro release of alveolar macrophage cytokines after the intratracheal instillation of bleomycin in rats. Characterization and kinetic studies. Am. Rev. Respir. Dis. 137:1135-1140.

Kehrer J.P., Witschi H. (1980): In vivo collagen accumulation in an experimental model of pulmonary fibrosis. Exp. Lung Research. 1:259-270.

Kelley J, (1990): State of the Art: Cytokines of the Lung. Am. Rev. Respir. Dis.141:765-788.

Khalil N, Bereznay O, Sporn M, Greenberg A.H., (1989): Macrophage Production of Transforming Growth Factor β And Fibroblast Collagen Synthesis In Chronic Pulmonary Inflammation. The Journal of Experimental Medicine. 170:727-737.

Khalil N, O'Conner R.N., Unruh H.W., Warren P.W., Flanders K.C., Kemp A, Bereznay O.H., Greenberg A.H., (1990): Increased Production and Immunohistochemical Localization of Transforming Growth Factor-β in Idiopathic Pulmonary Fibrosis. Am. J. Respir. Cell Mol. Biol. 5:155-162.

Khalil N, Greenberg A.H., (1991): The role of TGF-β in pulmonary fibrosis. Clinical applications of TGF-β. Wiely, Chichester (Ciba Foundation Symposium 157) p.194-211.

Khalil N, Whitman C, Zuo L, Danielpour D, Greenberg A, (1993): TGF-β secretion by corticosteriods in bleomycin induced pulmonary inflammation in the rat. J. Clin. Invest. 92(4):1812-8.

Khalil N, O'Connor R.N., Flanders K.C., Shing W, Whitman C.I., (1994): Regulation of type II alveolar epithelial cell proliferation by TGF-β during bleomycin induced lung injury in rats. Am. J. Physiol. 267(5 pt 1):L498-507.

Khalil N, O'Conner R.N., Flanders K.C., Unruh H, (1996): TGF-β_1, But Not TGF-β_2 or TGF-β_3, Is Differentially Present in Epithelial Cells of Advanced Pulmonary Fibrosis: An Immunohistochemical Study. Am. J. Respir. Cell Mol. Biol. 14:131-138.

Khalil N, Corne S, Whitman C, Yacyshyn H, (1996): Plasmin Regulates the Activation of Cell-associated Latent TGF-β_1 Secreted by Rat Alveolar Macrophages after In Vivo Bleomycin Injury. Am. J. Respir. Cell Mol. Biol. 15:252-259.

Korfhagen T.R., Swantz R.J., Wert S.E., McCarty M, Kerlankian C.B., Glasser S.W., Whitseett J.A., (1994):Respiratory epithelial cell expression of human transforming growth factor alpha induces lung fibrosis in transgenic mice. J. Clin. Invest. 93:1691-1699.

Kotani H, Newton P.B., Zang S, Chiang Y.L., Otto E, Weaver L, Blaese R.M., Anderson W.F., McGarrity G.J., (1994): Improved methods of retroviral vector transduction and production for gene therapy. Hum. Gene Ther. 5:19-28.

Kozak S.L., Kabat D, (1990): Ping-Pong Amplification of a Retroviral Vector Achieves High-Level Gene Expression: Human Growth Hormone Production. J. of Virology. 64-3500-3508.

Laiho M, Weis F.M.B. and Massague J, (1990): Concomitant loss of TGF-β receptors type I and II in TGF-β resistant cell mutants implicated both receptor types in signal transduction. J. Biol. Chem. 265(30):18518-18524.

Laitinen A, Laitinen L.A., (1994): Airway Morphology: Epithelium/Basement Membrane. Am. J. Respir. Crit. Care Med. 150:S14-17.

Laskin D.L., Pendino K.J., (1995): Macrophages and Inflammatory Mediators in Tissue Injury. Annu. Rev. Pharmacol. Toxicol. 35:655-77.

Lazo J.S., Hoyt D.G., (1990): The molecular basis of interstitial pulmonary fibrosis caused by antineoplastic agents. Cancer Treatment Reviews. 17:165-167.

Leiden J.M., (1995): Gene Therapy- Promise, Pitfalls, and Progonosis. The New England Journal of Medicine. 333:871-873.

Levine J.H., Moses H.L., Gold L.I., Nanney L.B., (1993): Spatial and temporal patterns of immunoreactive transforming growth factor 1, beta 2, and beta 3 during exicisional wound repair. Am. J. Pathol. 143:368-380.

Lynch C.M., Miller A.D., (1991): Production of high-titer virus-free retroviral vectors by cocultivation of packaging cells with different host ranges. J. Virol. 65:3887-3890.

Martinet Y, William N.R., Grotendorst G.R., Martin G.R., Crystal R.G.,(1987):Eagerated spontaneous release of platelet derived growth factor by alveolar macrophages from patients with idiopathic pulmonary fibrosis. New Engl. J. Med. 317:202-209.

Mason R.J., Shannon J.M., (1997): Alveolar Type II Cells. The Lung: Scientific Foundations Second Edition. Chapter 37:534-555.

Massague J. (1990): The Transforming Growth Factor-β Family. Annu. Rev. Cell Biol. 6:597-641.

Massague J, (1987): The TGF-β family of growth and differentiation factors. Cell. 49:437.

Massague J, Cheifetz S, Laiho M, Ralph D.A., Weis F.M.B., Zentella A, (1992): Transforming Growth Factor-β. Cancer Surveys. 12:81-103.

Mastrangeli, A, Canel C, Rosenfeld M.A., Perricaudet S, Perricaudet M, Pavirani A, Lecocq J.P., Crystal R.G., (1993): Diversity of airway epithelial cell targets for in vivo recombinant adenovirus mediated gene transfer. J. Clin. Invest. 91:225-234.

McCartney Francis N, Mizel D, Wong H, Wahl L, Wahl S, (1990): TGF-β regulates producton of growth factors and TGF-β by human peripheral blood monocytes. Growth Factors. 4:27-35.

McCartney Francis N, Mized D, Wong H, Wahl L.M., Wahl S.M., (1988): Transforming growth factor-beta as an immunoregulatory molecule. FASEB J. 2:A875. (Abstract).

McCray P.B., Armstrong K, Zabner J, Miller D.W., Koretzky G, Couture L, Robillard J, Smith A, Welsh M, (1995): Adenoviral mediated gene transfer to fetal pulmonary epithelia in vitro and in vivo.

Miller D.G., Adam M.A., Miller A.D., (1990): Gene transfer by retrovirus vectors occurs only in cells tht are actively replicating at the time of infection. Mol. Cell. Biol. 10:4239-4242.

Miller A.D., (1990): Retrovirus Packaging Cells. Human Gene Therapy. 1:5-14.

Moses H.L., Tucker R.F., Leof E.B., Coffey R.J., Halper J, Shipley G.D., (1985): Type β transforming growth factor is a stimulator and growth inhibitor, In: Feramisco J, Ozanne B, and Stiles C, (eds). Cancer Cells.3-7.

Moses H.L., Yang E.Y., Pietenpol J.A., (1990): TGF-beta stimulation and inhibition of cell proliferation :new mechanistic insights. Cell. 63:245-247.

Munaf A, Lemoine N.R., Ring C.J., (1994): The Use of DNA viruses as vectors for gene therapy. Gene Therapy. 1:367-384.

Nabel E.G., Plautz G, Nabel G.J., (1991): Gene Transfer into Vascular Cells. JACC. 17:189B-94B.

Nabel E.G., Gordon D, Yang Z.Y., Xu L, San H, Plautz G,. (1992): Gene transfer in vivo with DNA-liposome complexes: lack of autoimmunity and gonadal localization. Hum. Gene Ther. 3:649-56.

Nagy P, Schaff Z, Lapis K, (1991): Immunohistochemical detection of transforming growth factor-β1 in fibrotic liver diseases. Hepatology. 14:269-73.

Nathan C. Sporn M.B., (1991): Cytokines in Context. J. Cell Biol. 113:981-986.

Nathan C.F., (1987): Secretory Products of Macrophages. J. Clin. Invest. 79:319-329.

Neumann E, Schaefer-Ridder M, Wang V, Hofschneider P.H., (1982): Gene transfer into muse lyoma cells by electroporation in high electrical fields. EMBO J. 1:841-5.

Okuda S, Languino L.R., Ruoslahti E, Border W.A., (1990): Elevated expression of transforming growth factor-β and proteoglycan production in experimental glomerulonephritis: possible role in expansion of the mesangial extracellular matrix. J. Clin. Invest. 86:453-62.

Phan S.H.,(1995): New strategies for treatment of pulmonary fibrosis. Thorax. 50(4):415-21.

Postlethwaite A.E., Keski-Oja J, Moses H.L., Kang A.H., (1987): Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. J. Exp. Med. 165:251-56.

Roth J.A., Cristiano R.J., (1997): Gene Therapy for Cancer: What Have We Done and Where Are We going? Journal of National Cancer Institute. 89:21-39.

Roberts A.B., Sporn M.B., (1996): Transforming Growth Factor-β. The Molecular and Cellular Biology of Wound Repair. Ch. 8:275-308.

Roberts A.B., McCune B.K., Sporn M.B., (1992): TGF-β: regulation of extracellular matrix. Kidney Int. 41(3):557-9.

Roberts A.B., Heine U.I., Flanders K.C., Sporn M.B., (1990): TGF-β major role in regulation of extracellular matrix. Ann. N.Y. Acad. Sci. 580:225-32.

Roberts A.B., Sporn M.B., Assoian R.K., Smith J.M., Roch N.S., Wakefield L.M., Hein U.I., Loitta L.A., Falanga V, Kehrl J.H., Fauci A.S., (1986): Transforming growth factor type-beta: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc. Natl. Acad. Sci. USA. 83:4167-4171.

Ruden T.V., Stingl M.C., Wagner E, Zatloukal K, (1995): Generation of High-Titer Retroviral Vectors Following Receptor-Mediated Adenovirus-Augmented Transfection. Biotechniques. 18:484-485.

Saski H, Pollard R.B., Schmitt D, Suzuki F, (1992): Transforming Growth Factor-β in the Regulation of the Immune Response. Clinical Immu. and Immunopathology. 65:1-9.

Schmid P, Kunz S, Cerletti N, McMaster G, Cox D, (1993b): Injury induced expression of TGF-beta$_1$ mRNA is enhanced by exogenously applied TGF-beta. Biochem. Biophys. Res. Commun. 194:399-406.

Schneeberger E.E., (1997): Alveolar Type I cells. The Lung: Scientific Foundations Second Edition. Chapter 36:535-541.

Seyer J.M., Hutcheson E.T., Kang A.H., (1976): Collagen polymorphism in idiopathic chronic pulmonary fibrosis. J. Clin. Invest. 57:1498-507.

Sha X, Brunner A.M., Purchio A.F., Gentry L.E., (1989): Transforming growth factor beta1: importance of glycosylation and acidic proteases for processing and secretion. Mol. Endo. 3(7):1090-8.

Shaw R.J., Benedict S.H., Clark R.F., King T.E., (1991): Pathogenesis of Pulmonary Fibrosis in Interstitial Lung Disease. Am. Rev. Respir. Dis. 143:167-173.

Sime P.J., Xing Z, Grahm F.L., Csaky K.G., Gauldie J, (1997): Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J. Clin. Invest. 100(4) 768-76.

Simon R.H., Paine R, (1995): Participation of pulmonary alveolar epithelial cells in lung inflammation. J. Lab. Clin. Med. 126:108-18.

Snider G.L., Hayes J.A., Korthy A.L., (1978): Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin: Pathology and stereology. Am. Rev. Respir. Dis. 117:1099-108.

Snider G.L., (1983): Interstitial Pulmonary Fibrosis-Which Cell is the Culprit? Am. Rev. Respir. Dis. 127:535-9.

Sporn M.B., Roberts A.B., (1992): Transforming Growth Factor -β: Recent Progress and New Challenges. The Journal of Cell Biology.119:1017-1021.

Sporn M.B., Roberts A.B., (1991): Introduction : What is TGF-β? Clinical applications of TGF-β. Ciba Foundation Symposium. 157:1-6.

Sporn M.B., Roberts A.B., Wakefield L. M., De Crombrugghe B, (1987): Some recent advances in the chemistry and biology of transforming growth factor-beta. J. Cell Biol. 105:1039-45.

Starcher B.C., Kuhn C, Overton J.E., (1978): Increased elastin and collagen content in the lungs of hamsters receiving an intratracheal injection of bleomycin. Am. Rev. Respir. Dis. 117:299-305.

Temin H.M., (1989):Retrovirus vectors for Study of Biochemical Processes. Biochemistry International. 18:1-6.

Tucker R.F., Shipley G.D., Moses H.L., Holley R.W., (1984): Growth Inhibition from BSC-1 Cells Closely Related to Platelet Type β Transfroming Growth Factor. Science. 226:705-7.

Umezawa, H. et al, (1996): Antibiotic. Ser. A. 19:200.

Vanin E.F., Cerruti L, Tran N, Grosveld G, Cunningham J.M., Jane S.M.,(1997): The development of high-titer retroviral producer cell lines by using Cre-mediated recombination.

Varga J, Rosenbloom J, Jimenez S.A.,(1987):Transforming growth factor-beta causes a persistent increase in steady state amounts of type I and type II collagen and fibronectin mRNA's in normal human dermal fibroblasts. Biochemical J. 247:597-604.

Varmus H, (1988): Retroviruses. Science. 240:1427-1435.

Wahl S.M., (1992): Transforming Growth Factor Beta (TGF-β) in Inflammation: A cause and a cure. J. Clin. Immunology. 12:61-74.

Wahl S.M., McCartney-Francis N, Mergentagen S.E., (1989): Inflammatory and Immunomodulatory roles of TGF-β. Immunology Today. 10:258-261.

Wahl S.M., Hunt D.A., Wakefield L, McCartney-Francis N, Wahl L.M., Roberts A.B., Sporn M.B., (1987): Transforming growth factor beta (TGF-β) induces monocyte chemotaxis and growth factor production. Proc. Natl. Acad. Sci. 84:5788-5792.

Watson J.D., Gilman M, Witkowski J, Zoller M, (1992): Recombinant DNA. Second edition.

Wolff G, and Crystal R.G., (1997): Biology of Pulmonary Fibrosis. The Lung: Scientific Foundations Second Edition. Chapter 192:2509-2524.

Wrana J.L., Attisano L, Wieser R, Ventura F, and Massague J.(1994): Mechanisms of action of TGF-β receptor. Nature 370(6488):341-347.

Yamashita H, Dijke T, Franzen P, Miyasono K, Heldin C.H., (1994): Formation of hetro-oligomeric complexes of type I and type II receptors for TGF-β. J.Biol.Chem. 269:20172-20178.

Yingling J.M., Wang X, Bassing C.H., (1995): Signaling by the transforming growth factor-beta receptors. Biochimica et Biophysica Acta. 1242:115-136.

Ye S, Mittereder N, Wert S, Whitsett J.A., Wilmott R.W., Trapnell B.C., (1994): In vito evaluation of the safety of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA to the lung. Hum. Gene Therapy.5:731-744.

IMAGE EVALUATION
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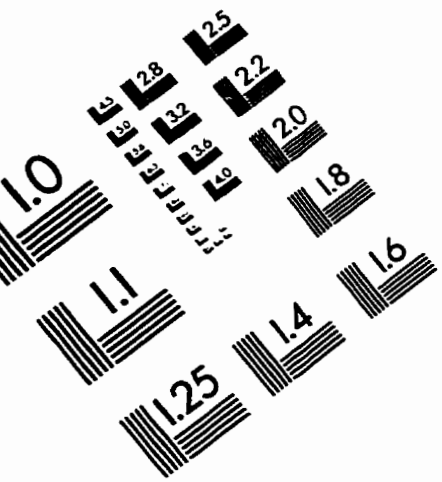

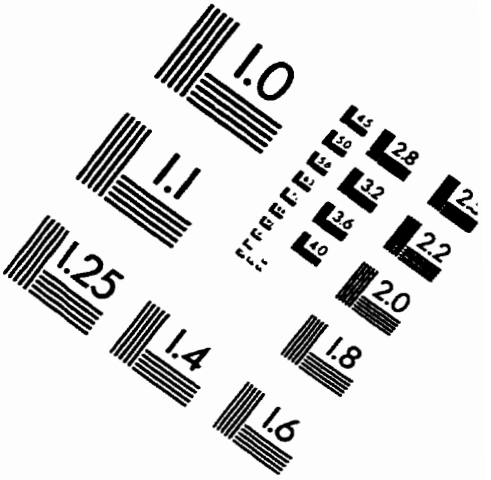

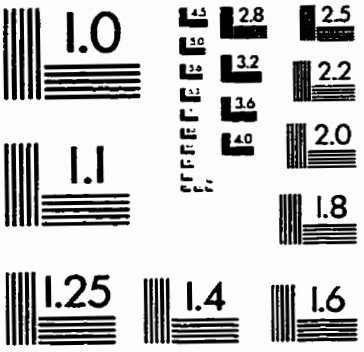

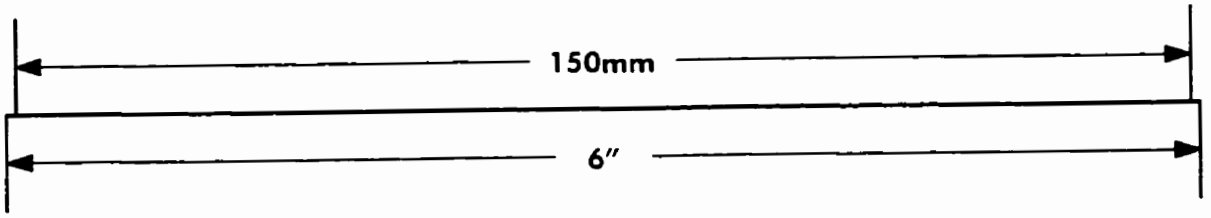

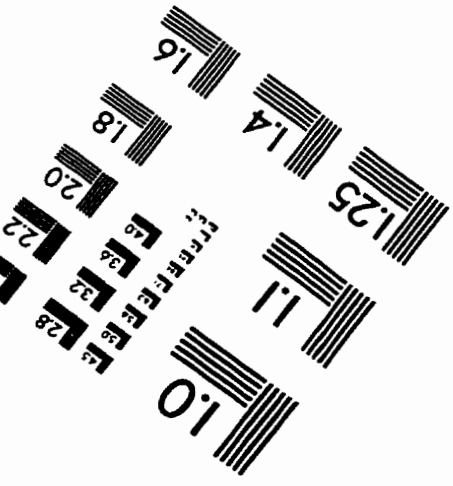

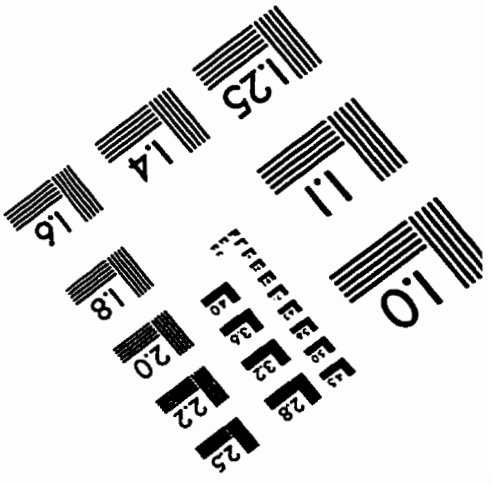

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