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Platelet-Derived Growth Factor and Pentoxifylline Modulation of Collagen Synthesis *In Vivo* and *In Vitro*.

by

Richard Allan Isbrucker

Submitted in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

at

Dalhousie University

Department of Pharmacology

Halifax, Nova Scotia

September, 1996

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Platelet-Derived Growth Factor and Pentoxifylline Modulation of Collagen Synthesis *In Vivo* and *In Vitro*"

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Dated: October 18, 1996

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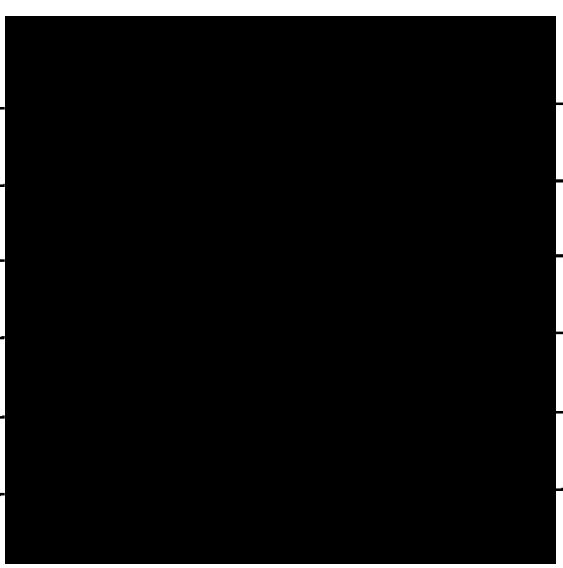

DALHOUSIE UNIVERSITY

DATE: 24 October, 1996

AUTHOR: Richard Allan Isbrucker

TITLE: "Platelet-Derived Growth Factor and Pentoxifylline Modulation of Collagen Synthesis *In Vivo* and *In Vitro*"

DEPARTMENT OR SCHOOL: Department of Pharmacology

DEGREE: Ph.D. CONVOCATION: May YEAR: 1997

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Abstract

Proliferation of fibroblasts and accumulation of extracellular matrix are two hallmarks of fibrotic liver disease. Hepatic stellate cells are the major collagen producing cells of the liver and are transformed into proliferative myofibroblasts following activation by cytokines. Whether myofibroblast proliferation and extracellular matrix production are regulated by the same cytokines is not known. An increase in total collagenous protein within the liver has been reported in several animal models of fibrosis including the yellow phosphorus induced swine model of hepatic fibrosis used in this thesis. Northern blot analysis of total RNA isolated from yellow phosphorus induced fibrotic pig liver show increased mRNA levels for collagens α1(I) and α1(III) at 8 and 12 weeks following the commencement of yellow phosphorus administration when compared to control pigs. Monocyte-conditioned medium from these fibrotic pigs induced collagen production by cultured porcine myofibroblasts which was reduced by pre-incubation with PDGF B/B antibody. Administration of pentoxifylline, concurrently with yellow phosphorus, to fibrotic pigs during the 8 to 12 week period decreased the mRNA levels for collagens α1(I) and α1(III). PDGF, which was previously shown to stimulate proliferation of non-confluent fibroblasts, is shown to induce both proliferation and collagen production in porcine myofibroblasts but that these two events are independent and can occur without the presence of the other. Pentoxifylline and its metabolite, M-1, inhibit PDGF-stimulated collagen production in cultured porcine myofibroblasts, but that these effects are not mimicked by elevations in intracellular cAMP. These results demonstrate the importance of PDGF in the development of liver fibrosis and provides evidence for the mechanism of pentoxifylline in reducing liver fibrosis.

Abbreviations

α1(I)	- alpha-1 segment of type I collagen
α1(III)	- alpha-1 segment of type III collagen
AA	- antibiotic/antimycotic
AP-1	- activator protein-1
BDL	- bile duct ligation
bp	- base pairs
BSA	- bovine serum albumin
cAMP	- cyclic adenosine monophosphate
cDNA	- complementary DNA
CPSR	- controlled-process serum replacement
CREB	- cAMP response element binding protein
cpm	- counts per minute
db-cAMP	- dibutyryl cyclic adenosine monophosphate
DMEM	- Dulbecco's modified Eagle's medium
ECM	- extracellular matrix
EDTA	- ethylenediaminetetraacetic acid
FP1	- Footprint 1
G3PDH	- glyceraldehyde-3-phosphate dehydrogenase
Grb2	- growth factor receptor binding protein-2
HEPES	- hydroxyethyl-piperazine-2-ethanesulphonic acid

IC_{50}	- inhibitory concentration, 50%
IL	- interleukin
i.p.	- intraperitoneal
kb	- kilobases
LAP	- liver activator protein
LPS	- lipopolysaccharide
M-1	- metabolite 1 of pentoxifylline
MAPK	- mitogen activated protein (MAP) kinase
MEK	- MAP kinase kinase
MCM	- monocyte conditioned medium
MO	- mineral oil
MOPS	- 3-N-morpholino-propanesulfonic acid
NECA	- n-ethylcarboxyamido adenosine
NF-1	- nuclear factor-1
NF-κB	- nuclear factor-kappa-B
NK cell	- natural killer cell
P-I-CP	- procollagen type I carboxy-terminal peptide
P-III-NP	- procollagen type III amino-terminal peptide
PAI-1	- plasminogen activator inhibitor 1
PBC	- primary biliary cirrhosis
PBS	- phosphate buffered saline
PDE	- phosphodiesterase

PDGF - platelet-derived growth factor

PG - prostaglandin

PKA - protein kinase A

p.o. - per os

PTX - pentoxifylline

RBC - Red blood cell (erythrocyte)

RER - rough endoplasmic reticulum

SDS - sodium dodecyl sulfate

SSC - standard sodium citrate

SSPE - standard sodium phosphate EDTA

sssDNA - sheared salmon sperm DNA

TAE - tris acetate EDTA

TCA - tricholoroacetic acid

TdT - terminal deoxynucleotidyl transferase

TE - tris EDTA

TGF - transforming growth factor

TIMP - tissue inhibitor of metalloprotease

TNF - tumour necrosis factor

Tx - thromboxane

YP - yellow phosphorus

Acknowledgments

This thesis could never have been written without the input and help from a lot of people over the past 5 years. First, I would like to thank Dr Theresa Peterson for her support and supervision during my graduate career, and for encouraging me to resume my studies when I was working as her technician. To Brenda White and Geriann Davidson I extend my many thanks for excellent technical assistance, patience and friendship; it has been a pleasure to work along side of you.

To Gordon Slysz, Rob Douglas, Stuart and Valerie Brown, and Lynn Thomas, I thank you all for your technical advice, many laughs (at my expense), and especially for your friendship. You've all helped to make these years a lot more enjoyable.

The anti-α-smooth muscle antibodies and the immunohistochemistry of the hepatic stellate cells was generously provided by Dr Geoff Rowden, with technical help from Pat Colp. Dr Michael Neumeister performed the bile duct ligations on the rats used in this thesis. Many thanks are also extended to the staff of the Dalhousie Animal Care Centre for their assistance and guidance in handling the pigs.

Many thanks are extended to the staff in the Pharmacology office: I would probably still be in an administrative line-up somewhere or trying to get a paper printed if it were not for your help and patience.

Finally, I would like to thank the Canadian Liver Foundation and the Anne van der Ree trust for financial support during the past 4 years.

Introduction

Liver diseases are the third leading cause of death due to illness in North America today (WHO 1995a; 1995b). The causes of fibrosis and cirrhosis are multiple and include alcoholism, congenital disorders (such as cystic fibrosis), autoimmune reactions, exposure to toxins or drugs, and viral or parasitic infections (Gressner and Bachem, 1994; Atterbury and Groszman, 1993). No cure exists for liver disease, and current treatments are aimed at delaying the progression of the fibrosis and to limiting exposure to the damaging agent (Wu and Danielsson, 1994; Atterbury and Groszman, 1993). Patients with advanced cirrhosis are usually referred for liver transplantation.

The mechanisms of initiation and progression of liver fibrosis are the subject of intense research. During the past 20 years a better understanding of the fibrotic process has been gained, largely due to the advances in molecular biology techniques. The current concepts of liver fibrosis are based on an injury occurring to the liver which triggers a chronic activation of the immune system. Immune mediators, such as cytokines, have the capacity to activate hepatic cells and initiate cellular proliferation and excess deposition of extracellular matrix proteins (Inuzuka *et al*, 1994; Friedman, 1993). Intercellular communications, which in the healthy liver maintain homeostasis, are altered during the fibrotic process and can lead to a perpetuation of the disease process (Gressner and Bachem, 1995; Pinzani, 1995; Friedman, 1993).

Liver physiology:

The liver is the largest organ in the human body and can weigh up to 5% of total body mass (Desmet, 1994). The size and structural organization of the liver is a reflection of its functional diversity and complexity. Blood is supplied to the liver by both the hepatic artery and portal vein (Hawker, 1993). These oxygen-rich and nutrient-rich sources of blood remain separate (Hawker, 1993) until entering the functional microvascular unit of the liver - the liver lobule. Each lobule is bordered by 3-6 portal triads which consist of the terminal branches of the hepatic artery and portal vein as well as the bile ducts (McCuskey, 1994; Jones, 1990). Blood enters the lobule at the portal triad and flows through sinusoids which merge at the centre of the lobule where they drain into the central vein for subsequent return to peripheral circulation (Hawker, 1993). Therefore, the branches of afferent vessels (hepatic artery and portal vein) and efferent vessels (central vein) are not joined directly, but rather are interdigitated and connected via the sinusoids (McCuskey, 1994; Hawker, 1993). This structural organization ensures all blood entering the liver has maximal exposure to liver cells (McCuskey, 1994). Due to this extreme vascularization and sponge-like architecture, the liver acts as a pool, or reservoir, for blood and contributes to the maintenance of blood volume and pressure (Desmet, 1994; Hawker, 1993; Jones, 1990).

Sinusoids are formed between plates of hepatocytes that radiate outward from the central vein (Hawker, 1993). Sinusoids are lined with endothelial cells (Kuiper *et*

al, 1994) and also contain hepatic stellate cells (Gressner and Bachem, 1995; Geerts *et al*, 1994), hepatic natural killer cells (Luo *et al*, 1995; Vanderkerken *et al*, 1995) and Kupffer cells (Kuiper *et al*, 1995). Plasma-contained nutrients are exchanged with hepatocytes by passing through the porous sinusoidal lining and the perisinusoidal space (also called the space of Disse). The space of Disse lies between the sinusoidal endothelial cells and the hepatocytes but is not an empty area: this site contains microvilli projections of the hepatocytes (Desmet, 1994; Hubbard *et al*, 1994), hepatic stellate cells (Pinzani, 1995; Geerts *et al*, 1994), and a low-density, basement membrane ECM (Pinzani, 1995; Gumucio *et al*, 1994)

Blood flow through the lobule is greatly affected during fibrosis. Cells lining the sinusoids are transformed in response to inflammatory mediators (Gressner and Bachem, 1995), hepatic stellate cells are believed to become contractile (Pinzani M, 1995; Desmet, 1994), and there is an accumulation of the ECM in the Disse space (Friedman, 1993). The resultant hindrance of blood circulation through the liver results in portal hypertension, the major complication of liver disease (Bosch *et al*, 1994; Atterbury and Grossman, 1993). These fundamental changes to the sinusoidal lining obstruct the exchange of nutrients and gases between the plasma and the hepatocytes, thereby affecting normal hepatic functions (Friedman, 1993; Bissell and Roll, 1990).

Hepatocytes:

Hepatocytes, or parenchymal cells, are responsible for the majority of hepatic functions including production of bile, glycogen storage, carbohydrate metabolism, lipid synthesis, formation of urea, clotting factors, albumin, lipoproteins and detoxification, or biotransformation, of exogenous compounds (Arias *et al*, 1994; Hawker, 1993). Hepatocytes are polygonal cells arranged in monolayer plates such that each cell borders on at least two sinusoids (Gumucio *et al*, 1994; Hubbard *et al*, 1994). Canaliculi are formed between adjacent hepatocytes into which they secrete bile (Gumucio *et al*, 1994). Seepage of bile into the sinusoids is minimized by the presence of tight junctions between adjacent hepatocytes (Spray *et al*, 1994). The interconnecting bile canaliculi converge at the bile ducts within the portal triads. Therefore, bile flow within the healthy lobule is in the opposite direction of the blood flow (Scharschmidt, 1990).

The hepatocyte is considered to be a polar cell with its 'external' (basal) surface exposed to the space of Disse and the 'internal' (apical) surfaces forming the bile canaliculi (Hubbard *et al*, 1994). Maintenance of this polarity is through cell-cell and cell-ECM contacts (Hubbard *et al*, 1994). Hepatocytes that are cultured on plastic dishes gradually lose their polarity and hepatocyte functions such as albumin production and cytochrome P450 expression (Gressner and Bachem, 1994). Contrary to this, hepatocytes grown in collagen gel or basement membrane matrix maintain their hepatocyte characteristics such as albumin production and cytochrome P450 activities

(Kono and Roberts, 1996; Dunn *et al*, 1992) as well as their cellular polarity (Hubbard *et al.*, 1994; Brill *et al.*, 1994; Lin and Bissell, 1993).

Although the hepatocyte responds to changes in the ECM (Lin and Bissell, 1993; DiPersio *et al*, 1991) and has the capacity to produce ECM proteins (Brenner *et al*, 1990), it is not considered to be a major contributor to this micro-environment (Gressner and Bachem, 1995; Pinzani 1995; Friedman, 1993). Localization of transcripts for collagen α2(I), α1(III) and α1(IV) by *in situ* hybridization reveal that non-parenchymal cells are the major source of hepatic collagen in bile duct ligated rat liver (Milani *et al*, 1990a) and in human fibrotic liver (Milani *et al*, 1990b). Also, mRNA levels from hepatocytes isolated from control, bile-duct ligated and carbon tetrachloride treated rats show no changes in collagen α1(I) or α1(III) mRNA levels whereas isolated endothelial and hepatic stellate cells from these same animals have greatly elevated levels of mRNA for these same collagen types (Maher and McGuire, 1990). Even in control rats both hepatic stellate cells and endothelial cells show greater expression of collagen α1(I) and α1(III) mRNA than expressed in the same number of hepatocytes (Maher and McGuire, 1990).

Several changes occur to hepatocytes during fibrosis that can perpetuate the course of the disease. Electron microscope examination of the Disse space reveal that hepatocyte microvilli, which allow for a maximal exchange of components between hepatocytes and plasma (Desmet, 1994; Hubbard *et al*, 1994), are lost in humans with

alcohol-induced liver fibrosis (Schaffer and Popper, 1963) and in rats with carbon tetrachloride-induced fibrosis (Martinez-Hernandez, 1985). The resultant decrease in the basal surface area of the hepatocyte is speculated to be in response to the changes in the surrounding ECM which occur during fibrosis (Friedman, 1993) and reduces the efficiency in which nutrients are exchanged with the plasma found in the Disse space (Scharschmidt *et al*, 1988). Additional hepatocyte functions that are altered in fibrosis include albumin production (Annoni *et al*, 1990), biliary secretion (Erlinger, 1994), and cytochrome P450 activity (George *et al*, 1995; Morgan and McLean, 1995; Kraul *et al*, 1991). For example, aryl hydrocarbon hydroxylase activity, a product of cytochrome P450 1A activity, is reduced in patients with liver disease (Peterson, 1991; Peterson and Williams, 1987; Brodie *et al*, 1981) as well as in the porcine model of hepatic fibrosis used in this study (Peterson *et al*, 1991). Damaged hepatocytes also release mediators that can further activate Kupffer cells and induce an inflammatory response (Greenwel *et al*, 1994). Kupffer cells cultured with different fractions of homogenized hepatocytes reveal that both the cellular membrane and cytosolic fractions are able to activate the Kupffer cells as measured by the release of pro-inflammatory cytokines and phagocytic activity (Green *et al*, 1996; Laskin and Pendino, 1995).

Sinusoidal cells:

There are four major cell types which line the sinusoids: endothelial cells, hepatic natural killer (NK, or pit) cells, Kupffer cells and hepatic stellate cells

(Desmet, 1994; Hawker, 1993). Although these four cell types each have specific functions, they also interact to maintain homeostasis in the perisinusoidal ecosystem and in the adaptation to physiological stresses (Desmet, 1994).

Endothelial cells:

Endothelial cells completely line the sinusoid from the portal region to the central vein (Desmet, 1994; Hawker, 1993). These cells form a barrier between the circulating blood and the parenchymal cells but allow for the flow of plasma into the Disse space through pores (fenestrae) which are arranged in sieve-like clusters penetrating through the endothelial cells (Kuiper *et al*, 1994; Arias, 1990). These sieve-plates provide a protective barrier to the hepatocyte by preventing any direct contact of larger particles, bacteria or viruses with parenchymal cells. Cytoskeletal rings comprised of actin and myosin control the size of the fenestrae (Arias, 1990) and respond to stimuli such as ethanol, nicotine and serotonin (Kuiper *et al*, 1994). The status of the underlying ECM is also known to affect the quantity and pore size of endothelial fenestrae *in vitro* (McGuire *et al*, 1992; Carley *et al*, 1988). Bovine adrenal cortex endothelial cells grown on a substratum of collagen type I, III, IV, V and laminin have approximately one third the number of fenestrations than when these cells are cultured on a matrix that does not contain collagen types I or III (Carley *et al*, 1988). This reduction in fenestrae number in the presence of collagens type I and III corresponds to the observed decrease in endothelial cell sieve plates that occurs during liver fibrosis in humans (Kuiper *et al*, 1994) where there is a significant

increase in the levels of these collagen types in the Disse space (Pinzani, 1995; Friedman, 1993).

Endothelial cells are also active in receptor-mediated endocytosis and processing of serum-born and extracellular matrix components (Kuiper *et al*, 1994; Smedsrød *et al*, 1990). Hyaluron and collagen receptors are involved in the attachment to, and phagocytosis of, degraded ECM, including procollagen terminal peptides (Kuiper *et al*, 1994; Smedsrød *et al*, 1990). Experiments with cultured liver cells show that endothelial cells endocytose ECM components at 10 times the rate of hepatocytes or Kupffer cells (Smedsrød *et al*, 1990). Although endothelial cells are able to synthesize collagens (Brenner *et al*, 1990; Friedman, 1990; Clement *et al*, 1986), the significance of their contribution to the ECM in health and fibrosis is not well known since immunohistochemical studies detect large amount of intracellular collagens which could be products of either endocytosis or production (Smedsrød *et al*, 1990).

Scavenger receptors on endothelial cells allow for the efficient endocytosis of serum-borne lipoproteins (VLDL, LDL, HDL) as well as chylomicrons (Kuiper *et al*, 1994). Whether these lipoproteins are actually metabolised by endothelial cells is in doubt since these cells do not express the enzymes for lipolysis (Kuiper *et al*, 1994). Current theory holds that lipoproteins and larger chylomicrons are endocytosed by endothelial cells, processed to a more bioavailable form and then released into the

Disse space for uptake by hepatocytes (Kuiper *et al*, 1994; Smedsrød *et al*, 1990).

Immunoglobulin G (IgG) and IgA complexes are also cleared from the circulatory system through endocytosis mediated by Fc receptors present on endothelial cells (Kuiper *et al*, 1994). The uptake of IgG complexes are believed to be able to stimulate the production and secretion of cytokines including TNFα, IL-1, IL-6 and HGF, as well as active eicosanoids (Smedsrød *et al*, 1990). Although Kupffer cells respond similarly to immunoglobulin complexes (Decker, 1990) the reaction of endothelial cells implicates them as a major contributor to the initiation and maintenance of the fibrogenic process (Kuiper *et al*, 1994).

Endothelial cells are important contributors to the maintenance of ECM within the space of Disse in both health and fibrosis (Kuiper *et al*, 1994). These cells actively produce collagens (Brenner *et al*, 1990; Friedman, 1990; Clement *et al*, 1986) and mRNA levels for collagens α1(I) and α1(III) are increased in carbon tetrachloride treated and bile duct ligated rats (Maher and McGuire, 1990). Endothelial cells also secrete fibronectin, undulin, and the collagenase activator plasminogen activator-1 (PA-1) (Kuiper *et al*, 1994).

Following activation by Kupffer cell-derived cytokines endothelial cells are capable of secreting endothelin-1, PDGF, TGFβ and TNFα, all of which contribute to the development and progression of the fibrotic process (Gressner and Bachem, 1996;

Friedman, 1993). For example, endothelin-1 induces the contraction of activated hepatic stellate cells (myofibroblasts) (Rockey and Weisiger, 1996; Kawada *et al*, 1993), and thereby alters the circulation within the lobule.

Hepatic natural killer cells (pit cells):

Hepatic natural killer (NK) cells are liver resident NK cells derived from the circulating leukocyte pool (Vanderkerken *et al*, 1993). These cells, also termed pit cells (Seaman, 1996), comprise less than 1% of the normal lever cell population (Luo *et al*, 1995). Hepatic NK cells are further differentiated in the liver to a more active, and lower-density (LD) form (Vanderkerken *et al*, 1995; 1993), a process that is partly dependant on interactions with Kupffer cells (Vanderkerken *et al*, 1995). Selective, toxic elimination of Kupffer cells *in vivo* with dichloromethylene diphosphonate results in a subsequent decrease in the LD-NK cell population (Vanderkerken *et al*, 1995) and incubation of high-density NK cells with Kupffer cell-conditioned medium increases the cytotoxicity of these NK cells (Vanderkerken *et al*, 1995). NK cells are important in the elimination of viral infected and tumour cells (Seaman, 1996) through the secretion of perforin, and TNFβ, and the induction of the apoptotic process (Seaman, 1996), but their role in the process of fibrosis and cirrhosis is unclear.

Reduced NK cell activity has been reported in patients with primary biliary cirrhosis (PBC) (Nishigaki *et al*, 1996; Matheson *et al*, 1989; James and Jonas, 1985). Since neither serum nor monocytes from PBC patients affects the spontaneous cell-

mediated cytotoxic activity of NK cells from control subjects (James and Jones, 1985) it is unlikely that the Kupffer cell/NK cell interactions are affected during PBC. PGE_2, which is elevated in liver fibrosis (Laskin and Pendino, 995; Decker *et al*, 1990), may be the cause of the reduced NK cell functions since it has been reported to inhibit *in vitro* NK cell activity (Nishigaki *et al*, 1996; Leung, 1989). Ursodeoxycholic acid, a bile salt used in the treatment of PBC (Hofmann, 1994; Poupon *et al*, 1987), improved the *in vitro* cytotoxic activity of NK cells from healthy volunteers and the *in vivo* activity of NK cells from patients with PBC (Nishigaki *et al*, 1996). Ursodeoxycholic acid was also found to reduce the PGE_2 production by peripheral blood mononuclear cells derived from healthy patients (Nishigaki *et al*, 1996) suggesting a possible mechanism by which this drug improves NK cell activity in PBC patients.

Kupffer cells:

Kupffer cells are liver-resident macrophages and as such act as scavengers for particulate matter like cellular debris, bacteria and viruses (Falasca *et al*, 1996). Kupffer cells reside in the sinusoidal lining attached to the luminal side of the endothelial cells but have the capacity to migrate within the liver in response to tissue damage (Laskin and Pendino, 1995; Kuiper *et al*, 1994). Administration of hepatotoxins, such as carbon tetrachloride (Edwards *et al*, 1993; Thompson *et al*, 1980) or acetaminophen (Laskin and Pilaro, 1986), induces the accumulation and localization of macrophages in the liver (Laskin and Pendino, 1995). The locations

and severity of subsequent liver injury from these toxins coincides with the numbers of Kupffer cells present (Laskin and Pendino, 1995; Laskin and Pilaro, 1986; Thompson *et al*, 1980). In the initiation of liver fibrosis activation of Kupffer cells occurs in response to cytosolic components, membrane fractions and eicosanoids released from damaged hepatocytes (Laskin and Pendino, 1995). Non-hepatic derived compounds such as immunoglobulin complexes, endotoxins and foreign antigens can also activate an inflammatory response by Kupffer cells (Green *et al*, 1996; Laskin and Pendino, 1995; Decker, 1990).

Although the Kupffer cell response is physiologically intended as a protective mechanism, the induction of inflammation can lead to greater tissue damage than is caused by the initiating factor. Suppression of *in vivo* macrophage function by hydrocortisone (Sudhir and Budhiraja, 1992) or gadolinium chloride (Iimuro *et al*, 1994; Edwards *et al*, 1993) reduces the severity of liver injury in response to hepatotoxins.

Kupffer cells, like all macrophages, mediate their cytotoxic and inflammatory effects through the release of reactive oxygen and nitrogen intermediates, cytokines, growth factors and hydrolytic enzymes (Kuiper *et al*, 1994; Decker, 1990). The secretion of ECM degrading enzymes (such as collagenase and elastase) allows for the infiltration of recruited macrophages and neutrophils and the release of reactive oxygen intermediates, providing these cells with cytotoxic capabilities (Decker, 1990). The

production of cytokines (colony-stimulating factors, Il-1, Il-6, TNFα, platelet-activating factor, TGFβ and PDGF) directs the recruitment of additional leukocytes (Decker, 1990) and promotes wound healing through a stimulation of ECM production and cell growth (Wahl, 1992; Raghow, 1991; Diegelman *et al*, 1990).

Chronic activation of Kupffer cells leads to the development of fibrotic lesions due to the long-term release of cytokines and growth factors that promote both cellular proliferation and ECM production. Regulation of Kupffer cell and cytokine activities would, therefore, provide an ideal action for pharmacological intervention of the fibrotic process.

Hepatic stellate cells:

Hepatic stellate cells (also called Ito cells, lipocytes, fat-storing cells of the liver or perisinusoidal cells) have a dual function of vitamin A storage and metabolism and of maintenance of the ECM of the Disse space in which these cells reside (Pinzani, 1995; Geerts *et al*, 1994; Friedman, 1993). In the healthy liver hepatic stellate cells have a quiescent phenotype: they have a low proliferation rate; retain retinoid stores in the form of lipid globules; and produce ECM components and modifying enzymes at basal levels (Gressner and Bachem, 1995; Pinzani, 1995; Geerts *et al*, 1994; Ramadori, 1991). Although hepatic stellate cells are not numerous in the liver (only 5-8% of the total liver cell population; Geerts *et al.*, 1994), these cells have microfilament-rich extensions that allow the cells to reach throughout the

perisinusoidal space. This location and circumsinusoidal spreading makes the hepatic stellate cells a strategic candidate for modulation of blood flow within the liver sinusoid (Pinzani, 1995; Geerts *et al*, 1994). Recent experiments on primary cultures of healthy hepatic stellate cells demonstrate their ability to contract in response to endothelin-1 (Pinzani *et al*, 1996; Rockey and Weisiger, 1996), thrombin, angiotensin-II, thromboxane A_2 (TxA_2) and prostaglandin F_2 (PGF_2) (Geerts *et al*, 1994; Kawada *et al*, 1993). PGI_2, PGE_2 and increased intracellular cAMP levels induce a relaxation of contracted hepatic stellate cells (Geerts *et al*, 1994). Hepatic stellate cells are also in direct contact with adrenergic nerves (Pinzani, 1995; Geerts *et al*, 1994; Ueno *et al*, 1988) suggesting a possible autonomic mechanism for regulating hepatic blood flow and total blood volume (Ueno *et al*, 1988).

Despite the seemingly innocuous nature of quiescent hepatic stellate cells, these cells become significant contributors to the pathology of liver fibrosis and cirrhosis following their activation (Gressner and Bachem, 1995; Pinzani, 1995). Hepatocyte necrosis or activation of Kupffer cells results in the production of factors, such as TNFα, which can activate quiescent hepatic stellate cells (Gressner and Bachem, 1995; Pinzani, 1995; Friedman, 1993). This activation causes a phenotypic change in the hepatic stellate cells: cytokine receptors are expressed (for PDGF, TGFβ) (Gressner and Bachem, 1995; Pinzani, 1995; Friedman, 1993; Heldin *et al*, 1991), α-smooth muscle actin and intermediate filaments appear in the cytoskeletal architecture and there is increased secretion of some ECM-degrading enzymes (eg; type IV collagenase)

(Gressner and Bachem, 1995; Pinzani, 1995, Friedman, 1993). The morphology of hepatic stellate cells also changes following activation; these cells begin to lose their retinoid stores, the rough endoplasmic reticulum becomes elongated and dilated, and the cells become more fibroblast-like in appearance (Pinzani, 1995; Geerts *et al*, 1994; Friedman *et al*, 1989). In this regard activated hepatic stellate cells are referred to as myofibroblast-like cells. Cytokines, such as PDGF or TGFβ, can further transform these cells into a proliferative myofibroblast which secrete high levels of ECM components, in particular collagens type I and III (Pinzani, 1995; Friedman, 1993). These features of fibroproliferation and excessive accumulation of ECM compounds are the hallmarks of liver fibrosis (Gressner and Bachem, 1995; Pinzani, 1995; Friedman, 1993).

Cultures of hepatic stellate cells isolated from healthy liver and grown on plastic or glass will also spontaneously transform into proliferative myofibroblast cells (Blazejewski *et al*, 1995; Pinzani, 1995; Ramadori, 1991). This *in vitro* transformation process corresponds with the loss of retinoid stores (Gressner and Bachem, 1995; Pinzani, 1995), expression of receptors to PDGF and TGFβ (Gressner and Bachem, 1995; Pinzani, 1995; Ramadori, 1991), increased production of the fibrillar collagen types I and III (Blazejewski *et al*, 1995; Gressner and Bachem, 1995; Pinzani, 1995), morphological changes into a fibroblast-like cell (Gressner and Bachem, 1995; Pinzani, 1995), the expression of α-smooth muscle actin (Gressner and Bachem, 1995; Pinzani, 1995; Geerts *et al*, 1994), and the increased expression of AP-1 and Sp-1 transcription

factors (Armendariz-Borunda *et al*, 1994). In all cellular aspects studied, it appears that the *in vitro* transformation of hepatic stellate cells reproduces the *in vivo* transformation that is seen in liver fibrosis (Blazejewski *et al*, 1995; Friedman *et al*, 1989) and makes cultures of hepatic stellate cells a viable model for studying specific facets of the transformation and fibrotic processes.

Extracellular matrix (ECM):

The ECM is a complex and dynamic structure that is found in all tissue types and is involved in cellular adhesion, regulation of tissue-specific gene expression, cytoskeletal structure and cellular growth (Rojkind and Greenwel, 1994; Lin and Bissell, 1993; Reid *et al*, 1992; Schuppan, 1990). In the liver, the major components of the ECM are the collagenous proteins (collagen types I, III, IV, V, VI; see table 1) which form 5 - 6% of the ECM dry weight in the healthy human liver (Gressner and Bachem, 1994) and approximately 1% in the healthy rat liver (Rojkind and Greenwel, 1994). The liver ECM also consists of numerous proteoglycans (such as fibronectin and laminin) and glycoproteins (such as dermatan sulfate, decorin and betaglycan) (Rojkind and Greenwel, 1994). These non-collagenous ECM components contribute only a small fraction of the total ECM mass (Gressner and Bachem, 1994; Rojkind and Greenwel, 1994).

Collagen Type	Normal Human Liver (mg/g wet weight)	Cirrhotic Human Liver (mg/g wet weight)	Fold Increase
I	2 (37%)	16 (53%)	8x
III	2 (37%)	8 (26%)	4x
IV	0.5 (9%)	5 - 7 (<23%)	10 - 14x
V	0.9 (16%)	5 - 7 (<23%)	5 - 8x
VI	0.01 (0.2%)	0.1 (0.3%)	10x
Total wet weight:	5.41	34.1 - 38.1	6 - 7x

Table 1: Mass, proportion (%) and fold increase of specific collagen types in normal and cirrhotic human adult liver. Note that the ratios between collagen types is also different in the cirrhotic liver than in the normal, healthy liver. Adapted from Gressner AM and Bachem MG (1994).

The ECM is not a static structure: it is constantly undergoing production and degradation by surrounding tissues (Rojkind and Greenwel, 1994). This allows for the modulation of the composition of the ECM enabling cells to adapt to physiological changes in their microenvironment, and provides a means of intercellular communication (Rojkind and Greenwel, 1994; Gressner and Bachem, 1994). ECM-specific cell surface receptors, called integrins, allow for an interaction between cell and matrix which can influence cellular differentiation, morphology, function, polarity and proliferation (Ruoslahti *et al*, 1994; Lin and Bissell, 1993). Integrins, which are transmembrane receptors (Rosales *et al*, 1995), are able to mediate these functions since they are in direct contact with cytoskeletal proteins (Lin and Bissell, 1993; Lampugnani *et al*, 1990) and also have the capacity to initiate signal transduction mechanisms such as the MAP kinase cascade (described in section on PDGF) (Chen *et al*, 1996).

In the healthy liver, as in most tissues, collagenous proteins comprise the majority of the ECM (table 1). Although hepatocytes and endothelial cells contribute to the production and maintenance of the sinusoidal ECM, hepatic stellate cells are the major producers of ECM proteins and modifying enzymes in both health and fibrosis (Pinzani, 1995; Friedman, 1993; Maher and McGuire, 1990). Fundamental changes occur to the ECM during fibrosis: the balance between production and degradation is disrupted (Arthur, 1994) and the composition of the matrix is altered (see table 1; Gressner and Bachem, 1994). In liver fibrosis there is a marked expansion of the

ECM due, mainly, to the increased production of collagenous proteins by activated hepatic stellate cells (Gressner and Bachem, 1995; Pinzani, 1995; Friedman, 1993). In contrast to this, liver collagenase activity is reduced in rats with carbon tetrachloride-induced cirrhosis (Montfort *et al*, 1990; Perez-Tamayo *et al*, 1987) and in baboons with alcohol-induced liver disease (Maruyama *et al*, 1982). This suppressed collagenase activity is likely due to the increased expression of collagenase inhibitors as is found in humans with liver fibrosis and cirrhosis (Benyon *et al*, 1996; Iredale *et al*, 1996; Li *et al*, 1994).

The ECM also serves as a reservoir for cytokines and growth factors including fibroblast growth factor (Brunner *et al*, 1991), TGFβ (Yamaguchi *et al*, 1990) and PDGF (Field *et al*, 1996). Association of growth factors with the ECM provides an extracellular store that enables their long-term bio-availability and protection from degradation (Rojkind and Greenwel, 1994). Although some cytokines, such as TGFβ (Yamaguchi *et al*, 1990) and fibroblast growth factor (Brunner *et al*, 1991), are retained in an inactive state, PDGF (Field *et al*, 1996) can bind to cell surface receptors without disassociation from the ECM. These extracellular stores of active factors also provide an immediate source for signalling migration, proliferation, and production of ECM components when the ECM has been damaged or following the enzymatic degradation that occurs during inflammation (Rojkind and Greenwel, 1994).

Collagens:

To date there are 13 known types of collagens in humans (Olsen, 1991), of which 5 can be found in the liver (Types I, III, IV, V, VI; see table 1) (Rojkind and Greenwel, 1994; Gressner and Bachem, 1993). All collagens are homo- or hetero-tripeptides assembled into characteristic triple α-helices of various lengths depending on the collagen type (Linsenmayer, 1991; Olsen, 1991). Fibrillar collagens (eg; types I, III) form fibre-like bundles that provide tensile strength to tissue (Rojkind and Greenwel, 1994). Non-fibrillar collagens (eg; type IV) form mesh-like bundles, or basement membrane, that can provide a scaffolding for other collagenous or non-collagenous ECM components (Rojkind and Greenwel, 1994).

All collagens undergo extensive post-translational modifications prior to assembly into the ECM (Olsen, 1991). The initial translation of collagen mRNA encodes for a preprocollagen peptide which contains a terminal hydrophobic signal sequence that is necessary for passage into the rough endoplasmic reticulum (RER) (Olsen, 1991) and is cleaved off immediately (Olsen, 1991) to form a procollagen peptide. During, or immediately following, the synthesis of the procollagen peptide many of the proline and some of the lysine amino acids are hydroxylated by prolyl or lysyl hydroxylases (Koda *et al*, 1994). Hydroxylation of proline is required for the formation of a stable α-triple helix (Pihlajaniemi *et al*, 1991). Collagens formed without the hydroxylation of prolines disassembles at approximately 27°C (Pihlajaniemi *et al*, 1991). At least 100 proline residues per α-helix must be

hydroxylated for collagen to remain stable at temperatures up to 40°C (Olsen, 1991). Hydroxylation of lysine in the collagen propeptides is necessary for the later glycosylation of the collagen protein but does not have an effect of the stability of the α-helix structure (Kellokumpu *et al*, 1994).

Prolyl and lysyl hydroxylases are RER-associated mixed function oxygenases that contain iron (Fe^{2+}) and copper (Cu^{2+}), respectively (Helaakoski *et al*, 1995; Kellolumpu *et al*, 1994). These metalloproteins require ascorbic acid as a co-factor (Tschank *et al*, 1994; Qian *et al*, 1993) and α-ketoglutarate and O_2 as substrates in the hydroxylation of proline and lysine residues (Olsen, 1991). Deficiencies in ascorbic acid (scurvy) results in insufficiently hydroxylated collagen that does not remain associated into fibrils at body temperature (Olsen, 1991). Active prolyl hydroxylase is a tetrameric ($\alpha_2\beta_2$) enzyme that is loosely associated with the RER membrane (Helaakoski *et al*, 1995; 1994). RER can contain inactive prolyl hydroxylase in the form of the β_2 peptide dimer (Helaakoski *et al*, 1995). The rate of collagen synthesis is proportional to the percentage of active enzyme within a cell (de Jong *et al*, 1991). Regulation of active prolyl hydroxylation activity is therefore dependant on the rate of formation of the α_2 peptides (de Jong *et al*, 1991). Production of active prolyl hydroxylase is susceptible to regulation by cytokines since both TGFβ and TNFα have been shown to increase its production whereas interferon-γ inhibits its production (Kawaguchi *et al*, 1992). Glycosylation of hydroxylated lysines by galactosyl-hydroxylysyl-transferase (Olsen, 1991) occurs prior to the assembly of the triple helix

(Olsen, 1991) but is not required for either proper collagen formation or secretion by the cells (Olsen, 1991). The functions of the glycosylation are not known but may be involved in the intracellular trafficking of the procollagens and/or integrin-collagen binding (Prockop and Kivirikko, 1995).

Proper folding of the collagen propeptides into the triple-helical structure commences with intra- and inter-peptide disulphide bond formation at the carboxy terminal ends of the collagen propeptides (Clive and Greene, 1994; Chessler and Byers, 1992; Dolz and Engel, 1990). Disulphide bonds are formed through the activity of the β_2 peptide dimer of prolyl hydroxylase (also called protein disulphide isomerase; Helaakoski *et al*, 1995; Clive and Greene, 1994). Assembled procollagens are transported from the RER to the golgi apparatus (Koda *et al*, 1994) and secreted from the cell via secretory vesicles (Olsen, 1991).

Prior to the assembly of secreted fibrillar procollagens (*ie:* collagens type I and III) into the ECM fibres, the amino- and carboxy- terminal ends are cleaved off of the protein by procollagen N- or C- proteinases (Rojkind and Greenwel, 1994). Procollagen proteinases are specific to the different genetic types of collagen (Olsen, 1991) and act extracellularly. The terminal polypeptides of collagens type I, III, IV and VI can be detected in serum by radioimmunoassay (Schuppan, 1991), but the value of these serum levels as markers of liver fibrogenesis is controversial. Although the levels of type I collagen within the liver are dramatically increased during fibrosis

(Gressner and Bachem, 1995; Pinzani, 1995). the serum levels of its carboxy-terminal peptide (P-I-CP) are also affected by collagen synthesis in bone and tendon; both of which are comprised mainly of collagen type I (Linsenmayer, 1991). The amino-terminal peptide of type III collagen (P-III-NP) has been shown to be elevated in patients with alcoholic fibrosis and cirrhosis (Schuppan, 1991; Bell *et al*, 1989; Torres-Salinas *et al*, 1986) but not in rats with dimethylnitrosamine or CCL_4-induced liver fibrosis (Ala-Kokko, 1992). In the latter the amino terminal propeptide of type IV (basement membrane) collagen (P-IV-CP) was significantly elevated in correlation with elevated mRNA levels for type IV collagen, whereas this correlation did not exist with type III collagen in these animal models (Ala-Kokko, 1992). The difficulty in interpreting P-III-NP levels exists because a portion of the amino-terminal peptides are not removed from all type III collagen molecules prior to assembly into the ECM (Schuppan, 1991); a mechanism believed to direct, or limit, the collagen fibril size (Linsenmayer, 1991; Vuorio and de Crombrugghe, 1990). Therefore, an elevation in serum P-III-NP levels can indicate active degradation of existing collagen fibrils and/or the synthesis of new type III collagen (Schuppan, 1991). Elevated serum procollagen terminal peptide levels can also be an indication of sinusoidal endothelial cell dysfunction since these cells are largely responsible for the clearance of these peptides from the serum (Smedsrød *et al*, 1990).

The function of the terminal collagen propeptide fragments is not limited to the assistance of α-helix structure formation. Procollagen peptides inhibit further collagen

production and collagen mRNA levels in fibroblasts (Vuorio and de Crombrugghe, 1990; Wu *et a*, 1986). P-I-CP fragments are internalised by fibroblasts and can bind to the promoter region of the type I collagen gene (Wu *et al*, 1991). Interestingly, cultured lipocytes (hepatic stellate cells) from bile duct ligated and CCl_4-treated rats do not exhibit reduced collagen protein or mRNA levels in the presence of P-I-CP compared to cultured lipocytes from normal rats (Ikeda *et al*, 1993). This suggests that procollagen terminal peptides play an important role in regulating the levels of collagen production, and that this inhibiting feedback response is affected during active fibrosis (Ikeda *et al*, 1993).

Collagen gene regulation:

Collagen α1(I):

In the healthy human (Milani *et al*, 1990b) and rat (Clement *et al*, 1986) liver type I collagen is found almost exclusively in the portal region and is absent from the Disse space. In the fibrotic and cirrhotic liver type I collagen becomes the most abundant ECM protein (table 1) and can be found in high quantities within the Disse space (Gressner and Bachem, 1994; Friedman, 1993; Milani *et al*, 1990b; Clement *et al*, 1986). Hepatic stellate cells which had not been producing collagen type I in the healthy liver start to express the genes for collagen α1(I) following activation (Pinzani, 1995).

Type I collagen is comprised of 2 molecules of α1(I) and 1 molecule of α2(I) collagen peptides (Linsenmayer, 1991). These two genes are located on chromosomes 17 and 7, respectively, in humans (Olsen, 1991; Bornstein and Sage, 1989). Several *cis*-activating elements have been located in the distal 5' region, the promoter region, first and fifth introns and the distal 3' flanking region of the collagen α1(I) gene (Brenner *et al*, 1994; de Crombrugghe *et al*, 1991; Vuorio and de Crombrugghe, 1990). These sequences are of both negative and positive regulatory elements such that the transcription rate of the collagen α1(I) gene is dependent on the summation of all inducing and inhibiting factors that are bound to their appropriate controlling elements.

The most proximal elements in the 5' untranslated region of the collagen α1(I) promoter contain 2 reverse CCAAT boxes each with adjoining or overlapping G+C-rich sequences (Houglum *et al*, 1994; Brenner *et al*, 1993; Nehls *et al*, 1992). The transcription factors NF-1 and Sp-1 are considered specific for these activating elements (Nehls *et al*, 1992). Recently, liver activator protein (LAP, HF-IL6 or C/EBPβ), a hepatocyte-enriched transcription factor, has been shown to interact at the same reverse CCAAT sites as NF-1 (Houglum *et al*, 1995). The binding of the NF-1 and Sp-1 are mutually exclusive within each binding domain such that only one transcription factor can bind to a site at a time (Nehls *et al*, 1992). The activity of this α1(I) promoter site is therefore dependent on the ratio of NF-1 and Sp-1 available for activation of the gene (Nehls *et al*, 1992). Selective mutation in either of the proximal

CCAAT boxes inhibits the binding capability of NF-1 and reduces the activity of the collagen α1(I) promoter (Karsenty and de Crombrugghe, 1990), suggesting that NF-1 is a transcriptional activator of type I collagen (de Crombrugghe *et al*, 1991). Selective mutation in either proximal GC-rich sequence areas inhibits the binding of Sp-1 and increases collagen α1(I) promoter strength (Karsenty and de Crombrugghe, 1990) suggesting that this sequence acts as an inhibitor of collagen type I transcription (de Crombrugghe *et al*, 1991).

Contrary results are found when a plasmid construct containing the proximal 5' promoter region of the collagen α1(I) gene is co-transfected with either the NF-1 or Sp-1 genes into *Drosophila melanogaster* cells, which lack both of these transcription activators (Nehls *et al*, 1992). In this system both Sp-1 (Li *et al*, 1995; Nehls *et al*, 1992) and NF-1 (Nehls *et al*, 1992) enhanced the collagen α1(I) promoter activity. Sp-1 was also found to be ten times stronger at activating this promoter region than NF-1 (Nehls *et al*, 1992). When both *trans*-activating factors are co-expressed in this system the activity of NF-1 is dominant (Nehls *et al*, 1992). Interpretation of these results is difficult because it does not take into account that there are additional Sp-1 binding sites located within the first intron of the collagen α1(I) gene (Brenner *et al*, 1994). Sp-1/Sp-1 interactions between the 5' promoter region and the first intron may alter the strength of the promoter activity (Courey *et al*, 1989) and may also be involved in regulating tissue specific expression of type I collagen (Brenner *et al*, 1994). Interestingly, Sp-1 binding activity is very low in normal liver and quiescent

hepatic stellate cells, but is greatly elevated in both fibrotic liver and in activated hepatic stellate cells where collagen α1(I) expression is also increased (Rippe *et al*, 1995; 1990). NF-1 levels are not greatly altered in liver fibrosis suggesting that Sp-1 may be the transcription factor involved in excess type I collagen accumulation in liver disease and that NF-1 may regulate constitutive (basal) levels of collagen expression (Armendariz-Borunda *et al*, 1994; Nehls *et al*, 1992).

TGFβ is a potent stimulator of collagen type I expression (Brenner *et al*, 1994; King *et al*, 1994; Hansch *et al*, 1994) but the mechanism(s) by which this cytokine activates the collagen gene is not well known. Plasmid constructs of the collagen α1(I) promoter region containing alterations in the NF-1 and Sp-1 proximal binding sites do not affect promoter activation by TGFβ (Brenner *et al*, 1994; Nehls *et al*, 1992). Interestingly, an NF-1 like binding sequence is located approximately 1600 bp upstream from the rat collagen α1(I) start site which is sensitive to induction by TGFβ (Ritzenthaler *et al*, 1993; 1991). A second TGFβ response element was also located in the distal 3' region of the collagen gene (Brenner *et al*, 1994). This TGFβ activation element is not sensitive to either NF-1 or AP-2, both of which have similar binding sequences (Ritzenthaler *et al*, 1991). Recently TGFβ was found to increase the activity of activator protein-1 (AP-1; Armendariz-Borunda, 1994), for which there are *cis*-activating elements in the collagen α1(I) gene (Brenner *et al*, 1994). Transfection experiments with plasmid constructs containing various lengths of the distal 5' region or the first intron of the rat collagen α1(I) gene showed that the deletion of an AP-1

site contained within the first intron reduced the expression by TGFβ but that the expression was not completely abolished (Armendariz-Borunda *et al*, 1994). These results suggest that the effects of TGFβ on collagen type I gene expression are partly mediated by AP-1 (Armendariz-Borunda *et al*, 1994).

Activation of the MAP kinase signal transduction cascade (see section on PDGF) has recently been shown to have differential effects on the expression of the collagen α1(I) gene in hepatic stellate cells (Davis *et al*, 1996). The terminal active enzymes of the cascade, MAP kinases, stimulate the expression of collagen α1(I) gene by an NF-1 and Sp-1 mediated effect (Davis *et al*, 1996). MAP kinases are also known to activate both *c-fos* and *c-jun* oncogenes that dimerize to form the AP-1 transcription factor (Malarkey *et al*, 1995; Seger and Krebs, 1995). Contrary to this effect, Raf kinase, an upstream enzyme involved in the MAP kinase cascade, inhibits collagen α1(I) gene expression (Davis *et al*, 1996; Slack *et al*, 1992) through a regulating site that is 1600 bp upstream from the NF-1/Sp-1 binding sites (Davis *et al*, 1996). The inhibitory effects of Raf are mediated through an uncharacterised 60-kDa protein that is present in high levels in activated hepatic stellate cells but is absent from quiescent stellate cells (Davis *et al*, 1996). These results indicate that the fundamental mechanisms controlling hepatic stellate cell expression of collagen α1(I) change following the transformation, or activation, of the stellate cells.

The regulatory mechanisms controlling collagen α1(I) gene expression are also cell type specific (Houglum *et al*, 1995). In studies using cells derived from transgenic mice the -0.44kb 5' region of the α1(I) gene, which contains the dual NF-1 and Sp-1 binding sites, was found to be critical for high levels of gene expression in hepatic stellate cells but not in skin or tendon fibroblasts (Houglum *et al*, 1995). Dermal and tendon fibroblasts did not require the -0.44kb region but did require the upstream -2300 kb region, which contains a TGFβ activating element and an AP-1 binding site, for proper expression of the α1(I) collagen gene (Houglum *et al*, 1995). Therefore the *cis*-acting elements required for high levels of collagen type I expression in the liver differ from those required in skin or tendon and make those fibroblast cells inappropriate for use in studying alterations of collagen production related to liver fibrosis.

Collagen α2(I):

As with collagen α1(I), the collagen α2(I) gene is also responsive to the *trans*-acting factors NF-1 (Rossi *et al*, 1988), and Sp-1 (Greenwel *et al*, 1995; Tamaki *et al*, 1995). Despite the similarities in activating mechanisms, these two collagen genes share little promoter region homology (de Crombrugghe *et al*, 1991).

Tamaki *et al* (1995) demonstrated that the Sp-1 transcription factor recognises three separate GC-rich sites that are located between the -270 to -310 bp region of the human fibroblast collagen α2(I) promoter. Plasmid constructs containing either

various lengths of the collagen α2(I) promoter or point mutations within the GC rich regions reveal that the proximal of the three Sp-1 binding sites conveys the strongest promoting influence whereas the most distal of these sites has almost no influence in controlling the expression of the reporter gene in this plasmid (Tamaki *et al*, 1995).

The collagen α2(I) promoter region does not have any reported NF-1/Sp-1 overlapping regions as are present in the α1(I) gene (de Crombrugghe *et al*, 1990). Although a CCAAT site exists in the proximal (-80 bp) region of the murine α2(I) gene, this site is not activated by NF-1 (de Crombrugghe *et al*, 1990; Rossi *et al*, 1988), but an uncharacterised binding factor recognises this site and promotes the expression of the murine α2(I) gene (de Crombrugghe *et al*, 1990; Maity *et al*, 1988). NF-1 does promote the activation of the murine α2(I) collagen gene by recognising a single site in the -310 to -290 bp region (Parés *et al, 1994;* Rossi *et al*, 1988).

The role of AP-1 in controlling the collagen α2(I) gene is currently unclear. TGFβ, which is a potent stimulator of collagen type I expression (Hansch *et al*, 1995; Brenner *et al*, 1994; King *et al*, 1994), is also known to induce the expression and activation of *c-fos*, *c-jun*, and *junB* which bind AP-1 sites (de Groot and Kruijer, 1990). It is likely that the influences of these oncogenes on collagen α2(I) expression in indirect since this gene does not have any AP-1 consensus sites (Chang and Goldberg, 1995).

Collagen α1(III):

Few studies have examined the transcriptional control mechanisms of the collagen α1(III) gene and, therefore, our understanding of its regulatory systems are limited. Early deletion analysis studies of the murine collagen α1(III) gene showed that the promoter region maintains a strong inhibitory mechanism on its expression (Murdyj and de Crombrugghe, 1988). Plasmid constructs containing various lengths of the α1(III) promoter fused to the chloramphenicol acetyltransferase gene show strong expression when only the proximal 200 bp are included (Murdyj and de Crombrugghe, 1988). The promoter activity is increased even further when only the proximal 80 bp of the α1(III) promoter are used, but are suppressed to basal levels when 400 bp or more are inserted into the plasmid.

Nuclear extracts from NIH 3T3 cells show that two different *trans*-acting factors bind to two sites within the proximal 200 bp of the murine collagen α1(III) gene (Ruteshouser and de Crombrugghe, 1989). Both of these factors positively regulate the α1(III) promoter and act independently of one another. Competitive inhibition assays using oligonucleotides with known transcription factor binding sequences reveal that the distal site (at -122 to -106 bp) is an AP-1 binding element (Ruteshouser and de Crombrugghe, 1989). The *trans*-acting factor that binds to the more proximal element (located at -83 to -61 bp) was determined not to be AP-2, NF-1 or Sp-1 (Ruteshouser and de Crombrugghe, 1989) and remains only partially characterised (Ruteshouser and de Crombrugghe, 1992).

Collagenases and tissue inhibitors of metalloproteases (TIMPs):

The levels of collagen within any tissue is dependent on its production and degradation rates. The constant turn-over of ECM components allows for tissue remodelling, repair and replacement of "aged" proteins. Although fibrosis is often considered an over-expression of collagen proteins, an inhibition of matrix degradation may also be a significant factor. ECM degrading enzyme activity is known to be significantly decreased in rats with carbon tetrachloride-induced fibrosis (Montfort *et al*, 1990; Perez-Tamayo *et al*, 1987), and the half lives of both collagens type I and III in these livers is doubled (Greenwel *et al*, 1994).

Matrix metalloproteases are a family of enzymes that are responsible for ECM degradation. These enzymes are comprised of: (1) interstitial collagenases, which degrade fibrillar type collagens (eg: collagen types I and III); (2) gelatinases (type IV collagenases), which degrade basement membrane and non-fibrillar collagens (eg: collagen types IV and V) and; (3) stromelysins which degrade collagen types III, IV and V and non-collagenous ECM proteins such as fibronectin and proteoglycans (Arthur, 1994). The activity of these enzymes is regulated by their transcription rates, activation rates of the pro-enzymes, and the presence of tissue inhibitors of metalloproteases (TIMPs) (Arthur, 1994; Denhardt *et al*, 1993; Shapiro *et al*, 1993).

Degradation of the ECM within the Disse space occurs during early stages of liver fibrosis (Arthur, 1994). Alterations in this ECM may affect hepatocyte function

(Lin and Bissell, 1993; DiPersio *et al*, 1991) and promote the activation of quiescent hepatic stellate cells (Gressner and Bachem, 1995; Pinzani, 1995). Activated stellate cells have been demonstrated to secrete 72 kDa Type IV collagenase and stromelysin-1 (Arthur, 1994). Growth factors and cytokines present during hepatic injury and inflammation (such as IL-1, TNFα and PDGF) are known to increase interstitial collagenase production by hepatic stellate cells and Kupffer cells (Arthur, 1994).

Hepatic stellate cells, but not Kupffer cells, are known to produce interstitial collagenase (Arthur, 1994) but the activity of this enzyme decreases in liver homogenates as liver disease progresses in carbon tetrachloride-induced liver fibrosis in rats (Montfort *et al*, 1990; Perez-Tamayo *et al*, 1987) and in baboon and human alcoholic liver diseases (Murawaki *et al*, 1994; Maruyama *et al*, 1982). The imbalance of ECM production *vs* degradation that is present during active fibrosis and the accumulation of fibrillar collagens in the space of Disse suggests that there is not an equivalent elevation of collagenase production, the collagenases are not being activated, and/or the enzyme is inhibited by TIMPs (Arthur, 1994). Although very few molecular studies of interstitial collagenase gene expression during liver fibrosis have been reported, *in vitro* studies using cultured hepatic stellate cells have shown that production of collagenase by these cells continues following their transformation into myofibroblasts (Montfort *et al*, 1990). After secretion by hepatic stellate cells, procollagenase requires the presence of the plasminogen/plasmin system for activation (Arthur, 1994), but increased production of plasminogen activator-inhibitor 1 (PAI-1)

by sinusoidal endothelial cells during inflammation is likely to inhibit the overall activity of the collagenase within the Disse space (Arthur, 1994).

TIMP-1 and TIMP-2 are both inhibitors of interstitial type collagenase. The primary source of these inhibitors in the liver are the hepatic stellate cells (Benyon *et al*, 1996), although hepatocytes and macrophages are also capable of producing TIMPs (Denhardt *et al*, 1993). Several studies have shown that TIMP levels are increased during chronic human liver diseases (Benyon *et al*, 1996; Murawaki *et al*, 1994; Li *et al*, 1993). Freshly isolated hepatic stellate cells from healthy liver express only low levels of mRNA for TIMP-1, but this increases as the cells are maintained in culture (Iredale *et al*, 1992). These studies suggest that the down-regulation of interstitial collagenase activity during active fibrosis is due to the inhibition of the activated enzyme by TIMPs.

Liver fibrosis:

Liver fibrosis is the outcome of a complex chain of events that has Kupffer cell activation and the release of active mediators as the key facets that determine the extent of the disease. Figure 1 outlines the major steps leading to the development of fibrosis. The noxious events that lead to the initiation of the fibrotic process are multiple and include exposure to hepatotoxins, infections and obstruction of bile formation (Friedman, 1993). Although the majority of *in vivo* models and human cases of liver fibrosis are considered to be derived from hepatocyte damage, this is not

the only mechanism that can cause an activation of Kupffer cells. Chronic injection of autologous serum into rats does not induce the direct damage of hepatocytes (Bhunchet *et al*, 1996; Paranetto and Popper, 1966), but rather causes the formation of immunoglobulin complexes that activate Kupffer cells (Bhunchet *et al*, 1996). Activation of Kupffer cells induces the release of mediators that have the potential to (a) induce further damage to cells and tissue; (b) further activate Kupffer cells and recruit additional leukocytes; and (c) activate non-leukocyte cells such as hepatic stellate cells (Gressner and Bachem, 1995; Pinzani, 1995; Friedman, 1993). Transformed (activated) hepatic stellate cells are proliferative and produce excess ECM components in response to Kupffer cell-derived cytokines (Gressner and Bachem, 1995; Pinzani, 1995; Friedman, 1993; Friedman and Arthur, 1989). The chronic exposure of the activated hepatic stellate cells to these factors leads to the formation of fibrosis (Gressner and Bachem, 1994; Friedman, 1993).

The link between proliferation of hepatic myofibroblasts and excess collagen accumulation *in vivo* is not well defined. Although activated myofibroblasts are known to have higher levels of collagen mRNA (Blazejewski *et al*, 1995; Armendariz-Borunda *et al*, 1994; Beno *et al*, 1993; Casini *et al*, 1991; Maher and McGuire, 1990; Nakatsukasa *et al*, 1990), the *in vivo* expansion of the ECM may be due more to the presence of a greater number of collagen-producing cells rather than the increase in the rate of actual collagen production per cell.

Figure 1: Sequence of events leading to the development of fibrosis. An injury or infection initiates the development of fibrosis by causing damage to hepatocytes. Kupffer cells become activated in response to the damaged or necrotic cells and promote the development of an inflammatory response: other leucocytes are recruited into the area and inflammatory mediators (such as cytokines, free radicals and eicosanoids) are released. These mediators have the potential to act on other sinusoidal cells and hepatocytes, injure neighbouring tissue, and activate the transformation of hepatic stellate cells. Transformed hepatic stellate cells (myofibroblasts) are proliferative in response to PDGF, and secrete elevated amounts of ECM components; two prominent features in the development of fibrosis.

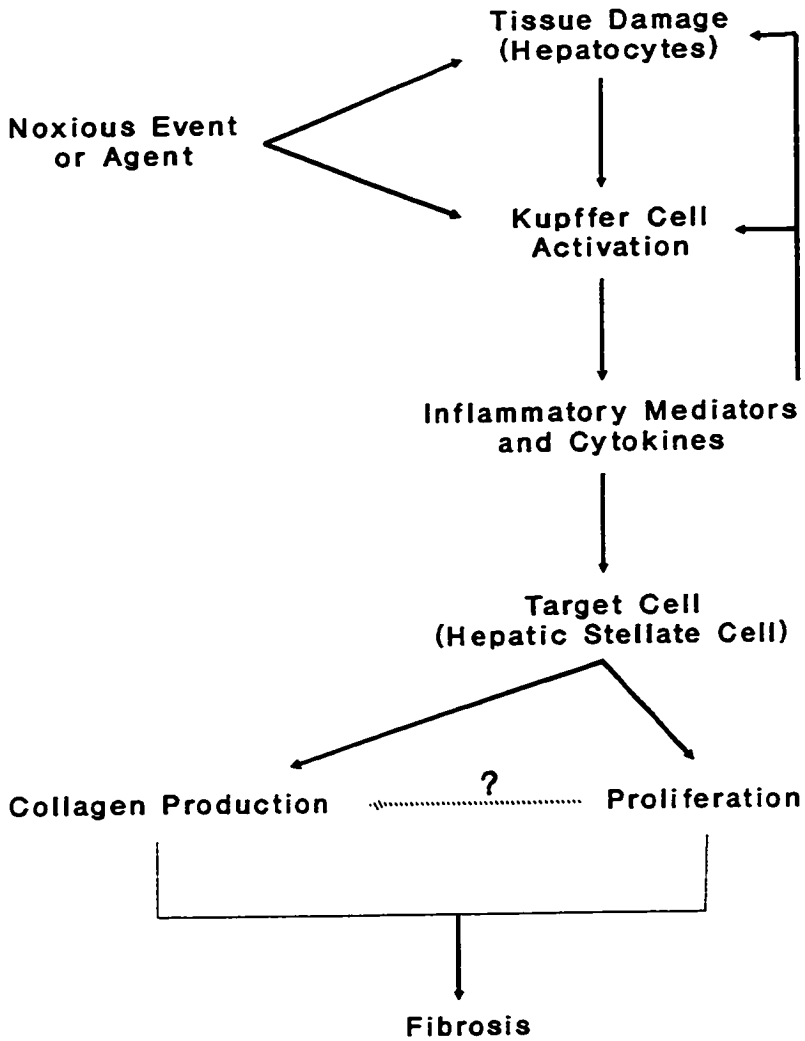

Figure 1: Linear arrangement of events leading to the development of fibrosis.

Of the cytokines that are produced by activated Kupffer cells TNFα, TGFβ and PDGF are considered the most important in terms of hepatic stellate cell transformation, proliferation and collagen production (Gressner and Bachem, 1995; Pinzani, 1995). TNFα and TGFβ promote the initiation of the hepatic stellate cell transformation into a transitional cell (Gressner and Bachem, 1995; Pinzani, 1995) which expresses receptors for PDGF (Friedman and Arthur, 1989) and TGFβ (Gressner and Bachem, 1995; Pinzani, 1995) and which further develop into myofibroblast-like cells. Collagen production by these fully transformed cells is elevated in response to TGFβ (Blazejewski *et al*, 1995). Although PDGF is known to induce the proliferation of myofibroblasts (Friedman and Arthur, 1989; Pinzani *et al*, 1989) several recent reports have provided conflicting evidence on the effect of PDGF on collagen production (Yi *et al*, 1996; Lepisto *et al*, 1995; Tan *et al*, 1995; Owens and Milligan, 1994; Pierce *et al*, 1994). This contradiction in results likely arises from differences in (a) cell types studied; (b) culture conditions; and (c) units by which collagen synthesis is measured.

Platelet-derived growth factor (PDGF):

Platelet-derived growth factor was originally described as a substance isolated from platelets that promoted the growth of cells of mesenchymal origin such as fibroblasts ((Kohler and Lipton, 1974), glial cells (Westermark and Wasteson, 1976) and smooth muscle cells (Ross *et al*, 1974). Further studies showed that this compound is also produced by monocytes/macrophages, fibroblasts and endothelial

cells (Meyer-Ingold and Eichner, 1996; Ross *et al*, 1990). PDGF is a dimeric protein composed of A and/or B chains linked by disulphide bonds (Heldin *et al*, 1993; Heldin, 1992). Therefore, three isoforms of PDGF exist: PDGF-AA, PDGF-AB, and PDGF-BB. Although all three isoforms are active (Heldin *et al*, 1993; Heldin, 1992), PDGF-AB is the prominent type produced in humans (Hammacher *et al*, 1988) and the PDGF-BB homodimer is the prominent isoform found in pig serum (Bowen-Pope *et al*, 1989).

PDGF exerts its actions on cells by binding to specific cell surface PDGF receptors (Claesson-Welsh, 1994). As with PDGF itself, the activated receptors are dimers of α and/or β chains (Claesson-Welsh, 1994; Heldin *et al*, 1992; Seifert *et al*, 1989). Dimerization of the receptor requires binding to the PDGF molecule (Bishayee *et al*, 1989). The PDGFα receptor recognises both the A and B chains of PDGF (Heidaran *et al*, 1990) whereas the β-receptor binds only the B chain (Claesson-Welsh, 1994; Heldin *et al*, 1992; Hart *et al*, 1988). The ratios of α to β receptors that are expressed on cells varies according to cell type (Heldin, 1992) and, therefore, the responsiveness of the cell to PDGF will be dependent on this ratio (Heldin, 1992; Ross *et al*, 1990). For example, human dermal fibroblasts express a 20:1 ratio of β to α receptors (Ross *et al*, 1990) and, therefore, do not respond as well to the PDGF-AA isoform as to the PDGF-BB isoform (Ross *et al*, 1990). Similar findings have been reported for cultured hepatic stellate cells (Wong *et al*, 1994; Heldin *et al*, 1991). The extent and specificity of the effect of PDGF is, therefore, dependent on both the PDGF

isoform secreted as well as the receptor types that are expressed (Claesson-Welsh, 1994; Heldin, 1992).

Activation of the PDGF receptors leads to the initiation of several signal transduction pathways of which the best studied is the MAP kinase cascade (for reviews see: Bornfeldt *et al*, 1995; Cano and Mahadevan, 1995; Malarkey *et al*, 1995; Seger and Krebs, 1995). Both the α and β PDGF receptors are transmembrane proteins with tyrosine kinase activities within the intracellular domains (Claesson-Welsh, 1994) that autophosphorylate the receptor upon dimerization (Kelley *et al*, 1991; Heldin, 1989; Keating *et al*, 1988). This phosphorylation allows for the interaction of the receptor molecule with a number of cytoplasmic and membrane-associated signal transduction proteins including growth factor receptor binding protein-2 (Grb2) (Malarkey *et al*, 1995; Yokote *et al*, 1994; Lowenstein *et al*, 1992).

Grb2 leads to the activation of the cytoplasmic Raf-1 kinase through an association with the mSos protein and the membrane-bound G-protein termed Ras-GDP (Malarkey *et al*, 1995; Seger and Krebs, 1995; Lowenstein *et al*, 1992). Grb2 binds directly with mSos and the resultant Grb2/mSos complex activate the nucleotide exchange activity on Ras to produce Ras-GTP (Aronheim *et al*, 1994). Cytosolic Raf will only bind to GTP-associated Ras and this interaction is necessary, but not sufficient, to initiate Raf kinase activity (Jelinek *et al*, 1996). Two poorly understood mechanisms of Raf activation involve a phospholipase C-directed activation or the

involvement of a protein termed 14-3-3 (Malarkey *et al*, 1995). The multiple mechanisms of Raf activation reflects the fact that a number of greatly differing signals can lead to the initiation of the MAP kinase cascade (Malarkey *et al*, 1995; Seger and Krebs, 1995).

Activation of Raf leads to the downstream activation of mitogen-activated protein kinase (MAP kinase) by an intermediate step involving MAP kinase kinase (Also termed MEK) (Peraldi *et al*, 1995). MEK represents a family of three highly homologous serine/threonine kinases that are themselves substrates of the serine/threonine kinase activities of Raf (Cano and Mahadevan, 1995; Malarkey *et al*, 1995). Activated MEK in turn phosphorylates and activates the MAP kinases ERK-1 and ERK-2 (Malarkey *et al*, 1995). Interestingly, another substrate for Raf is the inhibitory-κB (IκB) protein which maintains the NF-κB transcription factor in an inactive and cytosolic state (Li and Sedivy, 1993).

A large number of proteins and enzymes are phosphorylated and activated by the MAP kinases that control the cellular processes allowing for the transition out of the G_0/G_1 phase of the cell cycle (Seger and Krebs, 1995). Although ERK-1 and ERK-2 translate to the nucleus (Cano and Mahadevan, 1995), their substrates are both cytoplasmic and nuclear (Seger and Krebs, 1995). Transcription factors and nuclear proteins activated by MAP kinases include *elk-1*, *c-fos*, *c-jun*, *c-myc*, ribosomal S6 kinase and RNA polymerase II (Seger and Krebs, 1995). Cytoskeletal proteins such as

Tau and microtubule-associated proteins can be phosphorylated by MAP kinases implicating these enzymes in cytosolic reorganization and alterations in cellular morphology (Malarkey *et al*, 1995). Even proteins involved in the MAP kinase cascade, such as mSos, Raf and MEK can act as substrates for ERK-1 and ERK-2 (Malarkey *et al*, 1995), providing a feedback mechanism within the MAP kinase cascade.

The MAP kinase cascade in its own right provides a powerful signal transduction sequence to the PDGF receptor, but this is not its sole means of providing cytoplasmic signalling. Additional enzymes that become affiliated with the activated receptor include phospholipase C, phosphatidylinositol 3-kinase, Src tyrosine kinases, and GTPase-activating protein (Malarkey *et al*, 1995). Some of these enzymes may also activate the MAP kinase cascade (Marra *et al*, 1995; Choudhury *et al*, 1993) and can independently promote mitogenesis since cells expressing mutant PDGF receptors that lack the Grb2 binding site will still proliferate in response to PDGF (Malarkey *et al*, 1995).

Pentoxifylline (PTX):

Pentoxifylline (PTX; 1-(5-oxohexyl)-3,7-dimethylxanthine) is known to reduce the viscosity of whole blood (Ely, 1989, Ward and Clissold, 1987; Ehrly, 1975) and as such is used clinically to improve the capillary blood circulation in the treatment of claudication and cerebrovascular disorders (Samlaska and Winfield, 1994; Ward and

Clissold, 1987; Schubotz, 1976).

The mechanism by which PTX reduces blood viscosity is poorly understood (Samlaska and Winfield, 1994) but is likely due to the combined effects of this drug on the cells of the circulatory system. Studies show that PTX reduces the force required to pass whole blood (Ehrly, 1975), isolated erythrocytes (Smith *et al*, 1986) and isolated lymphocytes (Schmalzer and Chien, 1990) through a filter. PTX-treated red blood cells show increased deformability as observed by electron microscopy (Ely, 1988) and are able to pass through microcapillaries half of their size (Smith *et al*, 1986). Intracellular calcium, which hinders RBC deformability, is decreased in erythrocytes by PTX treatment (Seidler and Swislocki, 1992), possibly through the direct inhibition of the adenosinetriphosphatase action of the channels involved in the active transport of calcium (Seidler and Swislocki, 1992; Porsche and Stefanovich, 1978).

Neutrophil and monocyte deformability (Sonkin *et al*, 1992a), adhesion (Marzi *et al*, 1996; Salyer *et al*, 1990) and aggregation (Marzi *et al*, 1996) are also reduced in the presence of PTX. These effects on leukocytes may be of greater significance in reducing blood viscosity than the effects of PTX on RBCs since mononuclear cells are 700 times more effective at obstructing blood flow than are erythrocytes (Ely, 1989; Schmalzer and Chien, 1984). Additional properties of PTX that lead to improved microvascular circulation include reduced plasma fibrinogen (Jarrett *et al*, 1977),

decreased platelet aggregation and adhesion (Hammerschmidt *et al*, 1988) and reduced vasoconstriction (Sonkin *et al*, 1992b).

Recently PTX has been shown to block the production and action of tumour necrosis factor-α (TNFα) (Mattson *et al*, 1996; Kozaki *et al*, 1995; Yang *et al*, 1995; Schandene *et al*, 1992; Doherty *et al*, 1991; Salyer *et al*, 1990; Strieter *et al*, 1988). Peripheral blood monocytes exposed to lipopolysaccharide (LPS) *in vitro* secrete less TNFα in the presence of PTX (Mattson *et al*, 1996; Schandene *et al*, 1992; Doherty *et al*, 1991). Transcription rate of the TNFα gene (Schandene *et al*, 1992; Doherty *et al*, 1991), but not mRNA stability is affected by PTX (Doherty *et al*, 1991). Similar results are found *in vivo*: significantly lower serum TNFα levels are found in mice treated with PTX and challenged with LPS (Rice *et al*, 1994; Doherty *et al*, 1991); and perfusion of rat donor livers with a solution containing PTX prior to transplantation reduced Kupffer cell activation, superoxide anion release and suppressed the production of TNFα post-transplant (Kozaki *et al*, 1995). The actions of PTX also extend to the inhibition of the effects of TNFα since TNFα-treated monocytes have reduced chemotaxis and adherence *in vitro* following exposure to PTX (Salyer *et al*, 1990). The anti-TNFα activities of PTX have made this drug a good candidate for the treatment of septic shock, transplant toxicities and human immunodeficiency virus (HIV) infection (Mattson *et al*, 1995; Samlaska and Winfield, 1994; Bianco *et al*, 1991).

PTX may also be therapeutic in the treatment of excess scar and fibrotic tissue formation (Samlaska and Winfield, 1994). Normal human dermal fibroblasts have reduced production of collagen, glycosaminoglycan and fibronectin and increased collagenase activity when cultured in the presence of PTX (Berman *et al*, 1989). Fibroblasts derived from sclerodermal or morphoeal patches have rapid proliferation rates when cultured in the presence of fetal calf serum which is inhibited by the addition of PTX (Berman and Duncan, 1990). PDGF induced proliferation of normal human dermal fibroblasts *in vitro* is inhibited by PTX in a dose-dependent manner (Peterson *et al*, 1994; Peterson, 1993) indicating that the anti-fibrogenic actions of PTX are not limited to the inhibition of TNFα activity.

Several possible mechanisms exist by which PTX can block the cytokine-mediated effects of fibrogenesis. Direct TNFα receptor and PDGF receptor antagonistic effects of PTX do not seem likely since (a) TNFα type 1 and 2 receptor numbers and mRNA levels in normal human dermal fibroblasts are not altered in the presence of PTX (Berman *et al*, 1992) and (b) the binding of radiolabelled PDGF to dermal fibroblasts was not diminished by exposure to PTX (Slysz and Peterson, 1994). Also, PTX exhibits a selective inhibition of TNFα-induced adenylate synthetase production, but not TNFα-induced IL-6 production (Berman *et al*, 1992). Therefore, the effects of PTX on TNFα-mediated signalling must occur at a post-receptor event in the signal transduction of this cytokine.

Nuclear factor-kappa B (NF-κB) is a ubiquitous *trans*-acting regulator of gene expression for a broad range of proteins including such inflammatory cytokines as TNFα, IL-6 and IL-8 (Baldwin, 1996; Grilli *et al*, 1993). TNFα is considered to be the most potent stimulator of NF-κB activity (Baldwin, 1996; Schutze *et al*, 1995), but other factors, such as PDGF (Olashaw *et al*, 1992) and IL-1 (Grilli *et al*, 1993), are also known to induce NF-κB actions. Biswas *et al* (1993) demonstrated that PTX is an inhibitor of NF-κB action using transfections of plasmid constructs containing the HIV-1 long terminal repeat (LTR) region which is an NF-κB binding site. PTX inhibited NF-κB-induced expression of the LTR-associated enzymes to basal levels (Biswas *et al*, 1993) by preventing the binding of NF-κB to the LTR region (Biswas *et al*, 1993). Although NF-κB is involved in the regulation of cell growth (Baldwin, 1996; Grilli *et al*, 1993), it is unlikely that the inhibiting effects of PTX on collagen production is by a direct inhibition of NF-κB activity since there are no NF-κB binding sites reported for any fibrillar collagens.

Potential role of cAMP in PTX-interference of PDGF signal transduction:

Regulation of intracellular cAMP levels is another potential mechanism by which PTX could inhibit yellow phosphorus-induced fibrosis in pigs, PDGF-induced proliferation of fibroblasts, and serum-induced collagen production in fibroblasts. PTX is known to be a non-selective inhibitor of type III and IV cAMP phosphodiesterases (PDE) (Meskini *et al*, 1994). PTX has also been shown to increase the *in vitro* cAMP levels in both monocytes (Sinha *et al*, 1995; Bessler *et al*, 1986) and endothelial cells

(Yonemaru *et al*, 1991).

Increased intracellular cAMP has a broad range of physiological effects that could account for the effects of PTX in both *in vivo* and *in vitro* models of fibrosis. Contracted hepatic stellate cells (Geerts *et al*, 1994), endothelial cells and smooth muscle cells are relaxed when exposed to cAMP analogues or agents that induce the production of cAMP (Westendorp *et al*, 1994; Stelzner *et al*, 1989). Also, fenestral pore diameter is increased following elevation of intracellular cAMP levels (Arias, 1990). *In vivo*, these effects would equate to improved microvascular circulation within the diseased liver and possibly reduce the extent of hypoxia that is present in fibrosis and cirrhosis.

Cyclic-AMP has contrasting effects on proliferation in differing cell types and cell lines. PDGF- or serum-induced proliferation of rat mesangial cells (Chini *et al*, 1995; Li *et al*, 1995; Matousovic *et al*, 1995), PC-3 cells (Peraldi *et al*, 1995; Okutani *et al*, 1991) and cultures of freshly isolated rat hepatic stellate cells (Kawada *et al*, 1996) are inhibited in the presence of elevated cAMP. In contrast, Swiss 3T3 cells and thymocytes proliferate in response to increases in cAMP (Burgering *et al*, 1993; Rozengurt, 1986). Although the reason for this discrepancy is not clear, the differences in proliferation responses to cAMP is likely dependant on the cellular requirement for Ras protein-dependant activation of the proliferative response (Cook and McCormick, 1993).

Production of ECM components is also modified following an elevation in cAMP: both fibronectin production and mRNA expression have been clearly shown to be inhibited by elevated cAMP (Coats and Brecher, 1993; Miao *et al*, 1993); total collagen production by human intestinal smooth muscle cells (Perr *et al*, 1989) and rat hepatocytes (Andrabi *et al*, 1992) is inhibited following exposure of the cells to db-cAMP, forskolin, cholera toxin or isobutylmethylxanthine (IBMX); and Yamamoto *et al* (1994) demonstrated that elevating cAMP levels in cultured rat Schwann cells produced an increase in type IV collagen mRNA and a significant suppression in the mRNA levels for the fibrillar collagens types I and III. The influence of cAMP on collagen production by hepatic stellate cells or myofibroblasts is unreported. Hepatic stellate cell response to cAMP is not necessarily similar to that of Schwann cells since the genetic regulation and expression of the various collagen types is highly tissue specific (Houglum *et al*, 1995).

Several mechanisms exist by which cAMP could modulate the signal transduction of PDGF. Many genes, such as that for TNFα, contain negative regulatory elements that inhibit the gene transcription in response to elevations in intracellular cAMP (Lemaigre *et al*, 1993). These cAMP response elements are binding sites for the *trans*-acting cAMP response element binding protein (CREB), which is itself activated by protein kinase A (PKA) (Gonzalez and Montminy, 1983). The activation of PKA is itself mediated by elevations in cAMP (Häfner *et al*, 1994). Therefore, this mechanism of inhibition does not directly interfere with the signal

transduction sequences of PDGF, but rather reduces the ability of PDGF to promote the expression of those genes which are down-regulated by cAMP.

Recently, increases in cAMP were shown to inhibit growth factor-induced activation of MAPK in rat adipocytes (Sevetson *et al*, 1993) and mesangial cells (Li *et al*, 1995). This inhibition of MAPK activity occurs by the PKA-mediated inactivation of the Raf serine/threonine kinase (Cook and McCormick, 1993) and thereby blocks the PDGF receptor activation of MAPK via the Ras-Raf-MEK-MAPK pathway. Cyclic-AMP also attenuates the effects of PDGF by increasing the dephosphorylation of the activated PDGF receptor (Böhmer and Böhmer, 1996). This dephosphorylation process is mediated by the protein tyrosine phosphatase enzyme, which is thought to be activated by PKA (Böhmer and Böhmer, 1996). Therefore, cAMP-induced activation of PKA has the ability to inhibit PDGF-induced gene transcription at the receptor, the signal transduction and the gene promoter levels.

Pig and rat models of fibrosis:

Two different animal models of liver fibrosis are used in this thesis: a yellow phosphorus-induced porcine model of hepatic fibrosis; and a bile duct ligation rat model. In the former, chronic oral administration of yellow phosphorus to pigs results in the development of fibrosis by 8 weeks, bridging fibrosis by 12 weeks and cirrhosis with nodulations by 16 weeks, as determined by histology (Peterson, 1993; Peterson *et al*, 1991). Serum liver function tests remain normal in all pigs until the 10-12 week

period, after which serum albumin, γ-glutamyl transpeptidase, and aspartate aminotransferase become elevated in those animals receiving yellow phosphorus (Peterson *et al*, 1991). Aryl hydrocarbon hydroxylase activity in peripheral blood monocytes, hepatocytes, and Kupffer cells is significantly depressed by 8 weeks of yellow phosphorus treatment compared to control animals receiving mineral oil alone and remains at these reduced levels up to 16 weeks of phosphorus administration (Peterson *et al*, 1991). Monocyte conditioned medium (MCM) from fibrotic pigs stimulates the proliferation of cultured porcine dermal fibroblasts above the levels attained from the MCM of control pigs (Peterson and Neumeister, 1996) and this effect is present by 4 weeks of yellow phosphorus treatment. Total liver collagen content in fibrotic pigs is also elevated following 8 weeks of exposure to yellow phosphorus (Peterson and Neumeister, 1996; Peterson, 1993; Peterson *et al*, 1991). The mechanism by which yellow phosphorus induces fibrosis is unknown, but similar hepatic damage due to exposure to this element has also been reported in humans (Fernandez and Canizares, 1995; Greenberger *et al*, 1964; Fletcher and Galambos, 1963; Rubitsky and Myerson, 1949), rabbits (Mallory, 1933) and guinea pigs (Mallory, 1933).

Pentoxifylline, which was previously reported to inhibit PDGF-induced proliferation of fibroblasts (Peterson 1996, 1993; Peterson *et al*, 1994), reduces the extent of liver fibrosis in yellow phosphorus-treated pigs when administered concurrently with the phosphorus from either the commencement of the protocol

period at week 0 (Peterson, 1993), or following the onset of fibrosis at week 8 (Peterson and Neumeister, 1996). PTX also prevents the elevations in serum liver function tests and total liver collagen content (Peterson, 1993). MCM from pigs receiving both yellow phosphorus and PTX shows less stimulation of porcine fibroblast proliferation than similar pigs not receiving PTX (Peterson and Neumeister, 1996).

Unlike the porcine model of fibrosis described above, the bile duct ligated model in rats represents a cholestatic fibrosis induced by the mechanical blockage of bile flow from the liver (Kountouras *et al*, 1984; Trams and Symeonidis, 1957). Cirrhosis is established in these animals by 4 weeks following ligation (Kountouras *et al*, 1984; Trams and Symeonidis, 1957) and is apparent by the proliferation of bile ducts and bile duct epithelial cells (Tuchweber *et al*, 1996; Polimeno *et al*, 1995), nodulations, increase in portal and periportal connective tissues (Kountouras *et al*, 1984), and increased collagen deposition (Peterson and Neumeister, 1996; Scott *et al*, 1994). Serum alanine aminotransferase is elevated as early as 6 hours following ligation of the common bile duct (Tuchweber *et al*, 1996) and aspartate aminotransferase, γ-glutamyl transferase, bilirubin and alkaline phosphatase are all elevated up to 4 weeks (Peterson and Neumeister, 1996; Tuchweber *et al*, 1996). MCM from these fibrotic rats is not fibroproliferative (Peterson and Neumeister, 1996). Interestingly, inflammation is absent or mild in the bile duct ligated rat model of fibrosis (Scott *et al*, 1994; Milani *et al*, 1990; Kountouras *et al*, 1984).

Administration of PTX to bile duct ligated rats does not alter the outcome of the disease: with the exception of serum aspartate aminotransferase, serum liver chemistry is not reduced; and total liver collagen content is actually increased above those levels found in bile duct ligated rats not receiving PTX (Peterson and Neumeister, 1996). MCM from control, bile duct ligated, and PTX-treated bile duct ligated rats stimulate the uptake of tritiated thymidine into cultured fibroblasts equally, indicating the absence of monocyte-derived proliferative factors (Peterson and Neumeister, 1996).

Objectives

Previous reports from this lab have demonstrated that the total liver collagen content is increased in pigs with yellow phosphorus-induced liver fibrosis (Peterson, 1993) and in bile duct ligated rats (Peterson and Neumeister, 1996). Administration of PTX to fibrotic pigs reduced the accumulation of liver collagen (Peterson, 1993) but did not have this effect in the fibrotic rats (Peterson and Neumeister, 1996). The mechanism by which collagen accumulates in these livers and is reduced following PTX treatment is poorly understood. The first objective of this thesis is to examine the mRNA levels for the fibrillar collagens in these two models of fibrosis to determine if the accumulation of collagenous protein is due to increased expression of these genes. The second objective is to determine if PTX inhibits collagen production in the pig model of fibrosis by reducing the expression of the fibrillar collagen genes and to determine if there is any effect of PTX on collagen α1(I) mRNA in the rat model of fibrosis.

MCM from patients with liver disease (Peterson and Isbrucker, 1992) and from yellow phosphorus-treated pigs (Peterson and Neumeister, 1996) stimulates the proliferation of cultured fibroblasts. MCM from PTX-treated fibrotic pigs does not stimulate fibroproliferation (Peterson and Neumeister, 1996). The third objective of this thesis is to determine if monocyte-derived factors from the fibrotic pigs also stimulates the production of collagens *in vitro*, and whether administration of PTX to these pigs reduces the level of MCM-stimulated collagen production.

PDGF also stimulates the proliferation of cultured dermal fibroblasts (Peterson and Isbrucker, 1992) and pre-treatment of MCM obtained from patients with liver disease with anti-PDGF antibody reduces the ability of that MCM to stimulate fibroproliferation (Peterson and Isbrucker, 1992). The fourth objective of this thesis is to determine if PDGF can stimulate the production of collagen by cultured porcine myofibroblasts in the absence of proliferation and whether PDGF is important in the *in vitro* MCM-induced collagen production. PTX inhibits PDGF-induced proliferation of fibroblasts (Peterson *et al*, 1994; Peterson 1996, 1993). The fifth objective is to determine if PTX also inhibits PDGF-induced collagen production in cultured porcine myofibroblasts.

The mechanism by which PTX inhibits proliferation is not known, but previous studies have shown that M-1, the primary metabolite of PTX (Samlaska and Winfield, 1994), is also active in peripheral vascular disease (Ward and Clissold, 1987) and inhibits PDGF-induced fibroproliferation (Peterson, 1996). PTX is known to be a cAMP phosphodiesterase inhibitor (Meskini *et al*, 1994) and to increase intracellular cAMP levels (Sinha *et al*, 1995; Yonemaru *et al*, 1991; Bessler *et al*, 1986). The final objectives of this thesis are to determine if M-1 inhibits PDGF-induced collagen production and to determine if the effects of PTX on PDGF-induced proliferation and collagen production *in vitro* can be mimicked by the elevation of intracellular cAMP levels.

Materials and Methods:

Materials:

Ascorbic acid, bovine serum albumin, bromophenol blue, Sigma blend type F collagenase, type IV collagenase, controlled-process serum replacements types 1 (CPSR-1) and 2 (CPSR-2), dibutyryl-cAMP, ethidium bromide, ficoll, glycerol, histopaque, magnesium sulfate, magnesium chloride, trycine, pentoxifylline (PTX), polyvinylpyrrolidone, salmon sperm DNA, sarcosyl, sodium acetate, sodium citrate, trypan blue and xylene cyanol were all purchased from Sigma Chemical Company, St Louis, Missouri.

Ampicillin, casein-hydrolysed amino acids, cesium chloride, formamide, guanidine isothiocyanate, hydroxyethyl-piperazine-2-ethanesulphonic acid (HEPES), Luria-Bertani (LB) medium, β-mercaptoethanol, n-ethylcarboxyamidoadenosine (NECA), Nytrans nylon membrane, tannic acid and trichloroacetic acid (TCA) were obtained from ICN Biomedicals Inc., Montreal, Canada.

Antibiotic/antimycotic (AA; 0.1 mg/ml streptomycin, 100 U/ml penicillin and 0.25 U/ml amphotericin B), Dulbecco's modified Eagle's medium (DMEM). Lambda DNA *Hind III*-digested fragments, Liebovitz's L15 culture medium, random primers DNA labelling kit, phenol, porcine serum, restriction enzymes (*Aat II, Eco RI, Hind III*), RNA molecular weight ladder, RPMI 1640 culture medium, Tris and

trypsin/EDTA solution (0.05% trypsin, 0.53 mM EDTA·4Na) were purchased from Gibco/BRL Life Technologies, Ontario, Canada

Butanol, calcium chloride, chloroform, ethylene diamine tetracetic acid (EDTA), formaldehyde, glucose, isoamyl alcohol, isopropanol, mineral oil and sucrose were purchased from Fisher Scientific chemicals (Ontario, Canada).

Chloramphenicol, lysozyme, platelet-derived growth factors A/B and B/B. sodium dodecyl sulfate and triton X-100 were purchased from Boehringer Mannheim Canada Ltd, Laval, Quebec.

Ketamine hydrochloride was purchased from Rogar/STB Inc, London, Ontario. Atropine sulfate was purchased from Astra Pharma Inc, Mississauga, Ontario. Chromic (2-0), Dexon (1-0) and Novafil (1-0) sutures were purchased from Cyanamid Canada Inc., Montreal, Quebec.

Transformed *Eschericia Coli* containing the probes for human collagens α1(I) (probe HF677), α1(III) (probe HF934), α1(IV) (probe KK4), human fibroblast-type collagenase (probe pCllase 1), human tissue inhibitor of metalloproteinase 2 (TIMP-2; probe pSS38), human β-actin (probe HHCI89) and human glyceraldehyde-3-phosphate dehydrogenase (G3PDH; probe pHcGAP) were purchased from the American Type Culture Collection (Rockville, Maryland). A 45 base pair oligonucleotide probe for

collagen α1(I) was purchased from Genosys Biotechnologies Inc. (Woodlands, Texas).

The GeneClean kit, containing the sodium iodide, glass silica matrix and NEW wash buffer was purchased from Bio-101 Inc., La Jolla, California. Methyl-[^{3}H]-thymidine, and the 3'-end labelling kit which contained the terminal deoxynucleotidyl transferase (TdT) and sodium cacodylate buffer were purchased from Amersham Life Science (Arlington Heights, Illinois). [α-^{32}P]dATP, [α-^{32}P]dCTP, [^{3}H]-proline and Reflections autoradiography film were purchased from Dupont Canada, Mississauga, Ontario. Anti-α smooth muscle actin was from Dimension Laboratories Inc, Mississauga, Ontario. PDGF B/B antibody and PDGF B/B were purchased from R&D Systems, Minneapolis, MN. Molecular grade agarose was purchased from Bio-Rad Laboratories Ltd (Mississauga, Ontario, Canada). X-omat AR autoradiography film was purchased from Eastman Kodak, Rochester, NY. Sephadex (G-25 and G-50) prepacked columns were purchased from Pharmacia LKB Biotechnology, Upsala, Sweden. OptiPrep was purchased from Nycomed Pharma AS, Oslo, Norway. The Rapid RNA™ purification kit was purchased from Amresco Inc., Solon, Ohio.

Animal models:

All animal studies were approved by the Dalhousie University animal ethics committee. Female Duroc-Hampshire pigs, aged 5 weeks, were housed on wood shavings and straw and received water and pig chow *ad libitum*. Male Sprague-Dawley rats (150-200 g) were housed on clay chips and received water and rat chow

ad libitum.

Pig model of fibrosis:

The porcine model of fibrosis used is as previously described (Peterson *et al*, 1991). Yellow phosphorus (0.6 mg/kg) in mineral oil was administered *per os* 5 days per week to pigs commencing at 6 weeks of age. Control pigs received mineral oil without yellow phosphorus. Pentoxifylline (16 mg/kg) was administered *p.o.*, 5 days per week starting at week 8 of yellow phosphorus or mineral oil treatment.

Blood collection and surgical procedures were done under Halothane anesthetic following pre-operative sedation with ketamine (15 mg/kg, *i.m.*) and atropine (0.04 mg/kg *i.m.*). Whole blood, collected by subclavian puncture, was taken from all pigs at weeks 8 and 12 following the start of yellow phosphorus administration. Blood samples (20 ml) were taken for the preparation of monocyte-conditioned medium (see below). Wedge liver biopsies were taken following 8 and 12 weeks of yellow phosphorus treatment. Anaesthetized pigs were strapped to a surgical table in a supine position, shaved and cleaned for surgery. Biopsies were attained through a mid-line incision of approximately 12 cm commencing at the xiphoid region. A wedge of liver approximately 6 cm long and 2 cm wide was cut from the right main lobe and flash frozen in liquid nitrogen for later mRNA analysis. The liver incision site was closed with 2-0 chromic absorbable sutures and the abdominal incision closed with 1-0 dexon absorbable sutures (for the peritoneal membrane and muscular layers) and 1-0 novafil

non-absorbable sutures (for the dermal layer).

Rat model of fibrosis:

Liver sections were obtained from 6 rats that had undergone common bile duct ligation, or sham surgery, 4 weeks prior (Peterson and Neumeister, 1996). Briefly, animals were anaesthetized with halothane and access to the bile duct was gained via a mid-line abdominal incision. The bile duct was isolated from the surrounding connective tissue, ligated and cut. A sham operation was performed on control rats in which the abdomen was opened and sutured, as above, but the bile duct was not ligated or cut. Following recovery half of bile duct ligated rats received PTX (16 mg/kg/day *i.p.*) dissolved in saline. Four weeks following the bile duct ligation, animals were sacrificed, the livers excised and flash frozen in liquid nitrogen for use in mRNA studies.

Total RNA isolation from tissue:

This method was a modification of that described by Chomczynski and Sacchi, 1987. RNA was isolated from 100 - 200 mg of flash frozen liver sections. Samples were homogenized in 1 ml of Solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% w/v sarcosyl, 0.1 M 2-mercaptoethanol) using a 2 ml Potter Elvehjem type tissue grinder with a teflon pestle. Sodium acetate (0.1 ml, 0.2 M, pH 4.0) and 1 ml of Tris-saturated phenol were added and the sample vortexed. Chloroform:isoamyl alcohol (0.2 ml of 49:1) was added, the mixture vortexed and left on ice for 2 hours.

The samples were centrifuged at 3000 x g for 30 minutes and the aqueous phase removed, its volume measured, and transferred to a new tube. An equal volume of ice cold isopropanol was added to the aqueous phase and the sample gently mixed. The mixture was stored at -20°C for at least 1 hour. The sample was then centrifuged at 3000 x g for 5 minutes to pellet the RNA and the supernatant removed and discarded. The pellet was re-suspended in 0.3 ml of solution D, vortexed, 0.3 ml of isopropanol added, incubated for 1 hour at -20°C and centrifuged at 3000 x g for 5 minutes. The supernatant was discarded and the pellet suspended in 1 ml of ice cold 75% ethanol, placed on ice for 1 hour and centrifuged at 3000 x g for 5 minutes. This final wash step was repeated 3 times and the pellet allowed to dry at room temperature for 1 hour. The RNA pellet was dissolved in 0.5 - 1 ml of deionized, sterile water and the concentration determined by spectrophotometry (Milton Roy Spectronic 1001 plus UV/VIS Spectrophotometer, Rochester, New York).

Total RNA isolation from cultured myofibroblasts:

Cultured myofibroblasts at passage #6 were subcultured onto eight 79 cm^2 petri dishes and grown for 6 days in DMEM supplemented with AA and 20% CPSR-1 until confluence was attained (see section 2.13 for more details on cell culture conditions used). The media was removed from all plates and replaced with fresh media or fresh media containing either PDGF B/B (32 ng/ml), PDGF B/B (32 ng/ml) and PTX (539 μM), or PTX (539 μM) alone so that 2 petri dishes were used per factor tested. The cells were allowed to grow for an additional 24 hours at 37°C and then briefly rinsed

3 times in cold, sterile PBS.

The isolation of myofibroblast total mRNA was done using a purchased RNA purification kit. The methods followed were as outlined in the instructions for this kit. In this method, cells are lysed in a hypertonic solution at neutral pH followed by chloroform extraction to remove proteins. Samples are treated with an anionic detergent to denature RNA complexes and then acidified. The detergent is removed by the addition of low concentration salts and then the RNA is pelleted by centrifugation in high molecular weight polyol and high salt concentration. The resultant pellet is washed twice in 70% ethanol to remove residual salt, air dried, and resuspended in 20 µl TE buffer.

Determination of nucleic acid concentration:

An aliquot of nucleic acid solution was diluted 1:50 with distilled water and the absorbance was measured at 260 nm and 280 nm. The nucleic acid concentration was calculated by the equation:

Abs (260nm) x (1 cm/cuvette path length cm) x dilution factor x 0.04 µg/µl

= µg/µl nucleic acid.

The ratio of absorbance (260 nm):absorbance (280 nm) was also calculated and the sample either discarded or re-extracted if this ratio was less than 1.6.

Electrophoresis and northern blotting of isolated RNA:

Total RNA was separated by electrophoresis through a 3-4 mm thick denaturing agarose gel (40 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, 0.66 M formaldehyde, 1% w/v agarose, 2 μM ethidium bromide). RNA samples (10 μg) were diluted with an appropriate volume of loading buffer (50% v/v glycerol, 20% v/v formamide, 0.4% w/v xylene cyanol, 0.4% w/v bromophenol blue, 1 mM EDTA), heated to 95°C for 5 minutes and then placed on ice until loaded onto the gel. Electrophoresis was done at 50 volts in 1x MOPS buffer (10x MOPS = 4 M MOPS, 1 M sodium acetate, 200 mM EDTA, pH 7.0) for 30 minutes and then at 5 volts overnight (14-18 hours). The gel was carefully removed, photographed on a UV light box and rinsed twice for 20 minutes each in 500 ml of 10 x SSC solution (20x SSC = 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). The RNA was transferred to a charged nylon membrane by the Northern blotting method described in Maniatis et al (1982) using 10 x SSC solution. The membrane was then dried at room temperature and baked at 80°C for 2 hours.

Growth and isolation of plasmid derived probes:

Lyophilized bacteria were re-suspended in 1 ml of sterile Luria-Bertani medium supplemented with ampicillin (50 μg/ml). The cells were grown overnight in 25 ml of LB media supplemented with ampicillin at 37°C with constant shaking. A 5 ml aliquot of the cell suspension was removed, sterile glycerol added to a final concentration of 30%, and frozen at -70°C for storage. The remaining 20 ml of cell suspension was

added to 1 L of sterile M9 medium (22 mM sodium phosphate, 22 mM potassium phosphate, 8.5 mM sodium chloride, 18.7 mM ammonium chloride, 11.1 mM glucose, 10 g/L casein-hydrolysed amino acids, 1 mM magnesium sulphate, 0.1 mM calcium chloride, 50 μg/ml ampicillin, pH 7.4) in a 4 litre erlenmeyer flask and incubated, with vigourous shaking at 37°C. When the optical density of the solution reached 0.8 (at 600 nm; an indication that the culture was still in log phase growth; ref), 5 ml of chloramphenicol (34 mg/ml in ethanol) was added and the incubation continued, with shaking, overnight. The cells were then pelleted by centrifugation at 4000 x g for 5 minutes at 4°C and the supernatant discarded. The bacteria were re-suspended in 10 ml of TE buffer (10 mM tris, 0.1 mM EDTA, pH 7.5) and transferred into two polycarbonate ultracentrifuge tubes. The cells were re-pelleted by centrifugation at 4000 x g for 5 minutes at 4°C and the supernatant discarded. Each pellet was re-suspended in 4 ml of cold sucrose (10% in 50 mM Tris, pH 8.0) and 0.5 ml of lysozyme (30 mg/ml) was then added to each tube to digest the bacterial cell walls. The solution was incubated on ice for 10 minutes and then 1 ml of EDTA (0.5 M, pH 8.0) was added to each tube, mixed, and incubated a further 5 minutes on ice. Triton X-100 (2.5 ml of 1% in water) was added to each tube, mixed and placed in a 37°C water bath for 2 - 5 minutes. The mixture was centrifuged at 192,000 x g for 30 minutes at 4°C, the supernatant collected and the total volume brought to 20 ml with sterile distilled water. 19.0 g of cesium chloride was added, dissolved by gentle mixing, and 400 μl of ethidium bromide (10 mg/ml) added. The solution was transferred to two heat sealable (crimp-top) centrifuge tubes, topped up with liquid

paraffin, sealed and centrifuged at 225,000 x g overnight. The centrifuge was stopped without a brake. The presence of two distinct DNA bands was checked using a UV light and the lower one removed by syringe and needle inserted through the side of the tube. To each millilitre of solution removed, 3 ml of TE buffer was added. n-Butanol (4 ml) was added, gently mixed and centrifuged at 1500 x g for 5 minutes to separate the phases. The top (n-Butanol) phase was removed and discarded and the aqueous phase re-washed with 4 ml of n-Butanol until all of the orange colour was removed. The aqueous phase was transferred to a 50 ml polypropylene centrifuge tube and 8 ml of cold ethanol was added for each ml of solution removed from the seal-top centrifuge tube. This was placed on ice for 20 minutes and centrifuged at 12 000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended in 400 µl of TE buffer and transferred to a 1.5 ml centrifuge tube. Tris-saturated phenol (0.5 ml) and 0.5 ml of chloroform were added, mixed and centrifuged for 2 minutes. The aqueous phase was transferred to another 1.5 ml centrifuge tube and 50 µl of sodium acetate (3 M) was added, mixed, 1 ml of ethanol added, mixed, and placed on ice for 10 minutes. The tube was centrifuged for 5 minutes and the supernatant discarded. The pellet was washed twice in 80% ice cold ethanol followed by centrifugation for 5 minutes. The pellet was air-dried and re-suspended in 250 - 500 µl of TE buffer. The concentration of the plasmid was determined by spectrophotometry and the remaining sample was stored at -20°C until use.

Removal and purification of probe from plasmid:

Complementary (cDNA) probes were cut from their plasmids using the restriction enzyme(s) indicated in table 2. For restriction enzyme digestion 10 μg of plasmid was transferred into a 0.5 ml centrifuge tube, 2 μl of 10x restriction enzyme reaction buffer (for *Eco RI*: 0.5 M tris-HCl (pH 8.0), 0.1 M magnesium chloride, 1 M sodium chloride; for *Pst I, Xba I:* 0.5 M tris-HCl (pH 8.0), 0.1 M magnesium chloride, 0.5 M sodium chloride; for *Aat II, Hind III, Sma I:* 0.2 M tris-HCl (pH 8.0), 50 mM magnesium chloride, 0.5 M potassium chloride) and 20 units of each enzyme was added and the total volume brought to 20 μl with sterile distilled water. The solution was gently mixed and centrifuged to bring the contents to the bottom of the tube. Digestions were carried out at 37°C for 1 hour. The reaction was stopped by the addition of 2 μl EDTA (0.25 M, pH 7.5) and placing the tube on ice. Following restriction digestion, the probes were separated from the linear plasmid by electrophoresis through a 1% non-denaturing agarose gel (40 mM tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2, 1% w/v agarose, 2 μM ethidium bromide). The electrophoresis was allowed to proceed at 25 - 50 volts until the nucleic acid bands were discernable from one another when viewed under ultraviolet light. The molecular weights of each digested plasmid DNA band was determined by comparison to molecular weight standards (lambda DNA *Hind III* fragments) run in the same gel. The band containing the probe was cut from the gel using a scalpel, the weight of the

Probe	Clone name	Insert size (kb)	Restriction Enzyme
collagen α1(I)	Hf677	1.8	*Eco RI*
collagen α1(III)	Hf934	1.3	*Aat II, Hind III*
collagen α1(IV)	KK4	1.7	*Pst I*
collagenase	pCllase 1	2.054	*Hind III, Sma I*
β-actin	HHCI89	1.1	*Eco RI*
TIMP-2	pSS38	0.791	*Pst I*
G3PDH	pHcGAP	1.2	*Pst I, Xba I*

Table 2. Clone name, insert size and restriction enzyme(s) used to digest the cDNA probes from their plasmids. All probes are of human origin. TIMP-2, tissue inhibitor of metalloproteinase-2; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

gel determined and the piece placed in a 6 ml polypropylene tube. The probe was purified from the gel using a Geneclean™ Kit. The gel piece was suspended in 2.5 - 3 times the volume of sodium iodide (6 M) and the tube incubated at 55°C until the agarose melted. The tube was inverted several times and 5 µl of glass silica matrix suspension was added. The tube was inverted several times and left on ice for 10 minutes with the contents mixed every 2 minutes. The tube was centrifuged at 200 x g for 1 minute and the supernatant discarded. The pellet was re-suspended in 500 µl of NEW™ wash buffer and transferred to a 1.5 ml centrifuge tube. The tubes were centrifuged for 10 seconds at 14000 x g and the pellet washed three more times in cold wash buffer. After the third wash the pellet was re-suspended in 10 µl of TE buffer. The tube was incubated at 50°C for 3 minutes, centrifuged at 14000 x g for 30 seconds to pellet the silica matrix and the supernatant transferred to a new 0.5 ml centrifuge tube. The pellet was re-suspended in TE buffer a second time, incubated at 50 °C for 3 minutes, centrifuged for 30 seconds at 14000 x g and the supernatant was collected and pooled together. The concentration of the probe was determined by spectrophotometry by the method in section 2.3.

Labelling of plasmid-derived probes:

Plasmid-derived probes were labelled with [α-^{32}P]dCTP using a random primers DNA labelling kit. A 25 ng aliquot of probe was diluted to 23 ul with distilled water and denatured by heating for 5 minutes in a boiling water bath. Deoxyribonucleoside triphosphates (2 µl each of dATP, dGTP, dTTP; 0.5mM in 3 mM tris, 0.2 mM

EDTA), 5 µl [α-^{32}P]dCTP (3000 Ci/mmol, 10 µCi/µl), 15 µl random primer mixture, and 1 µl of klenow fragment (large fragment of DNA polymerase I) were added to the denatured probe and incubated at 25°C for 1 hour. The labelling reaction was stopped by cooling on ice and adding 5 µl EDTA (0.5 M, pH 7.5).

Labelling of oligonucleotide probe:

A short, 45 base pair, oligonucleotide probe specific for collagen α1(I) mRNA was derived from the published sequence of segment 3 of rat collagen α1(I) mRNA (Genovese *et al*, 1984):

3'- tatgttttggtggttctggagggcggacgggtagtagctacaccg - 5'

This 45 base pair region exhibits 100% homology with known regions within the human and mouse collagen α1(I) gene, 84% homology with chicken collagen α1(I) mRNA, and less than 75% homology to known sequences for non-α1(I) collagens as determined through the National Institutes of Health (Bethesda, Maryland) gene sequence databank. The oligonucleotide probe was 3'-end labelled with [α-^{32}P]dATP using a terminal deoxynucleotidyl transferase (TdT) enzyme. A 5 pmol aliquot of probe was transferred to a 0.5 ml centrifuge tube, along with 5 µl of cacodylate buffer, 12 µl [α-^{32}P]dATP (3000 Ci/mmol; 10 mCi/ml), 10 units TdT and sufficient distilled water to bring the final volume to 50 µl. The contents of the tube were gently mixed and the labelling allowed to proceed at 37°C for 1.5 hours. EDTA (5 µl, 0.5M, pH 8.0) was added to the tube to stop the reaction, and the probe was purified from the unincorporated nucleotides by separation through a Sephadex column.

Purification of labelled probes:

Plasmid-derived cDNA probes were purified by eluting through a Sephadex G-50 column. Pre-packed columns were re-suspended by repeated inversion and the Sephadex packing left to settle for 5-10 minutes. The column was then drained and equilibrated with 2 washes of TE buffer. The column was then centrifuged at 500 x g for 4 minutes. Labelled probe was carefully placed onto the top of the column and a 1.5 ml centrifuge tube was placed under the column to collect the eluent. The column was then re-centrifuged at 500 x g for 5 minutes and the purified probe recovered from the 1.5 centrifuge tube.

The labelled oligonucleotide probe was purified by eluting through a Sephadex G-25 column. The pre-packed column (0.4 x 1.2 cm) resin was resuspended by gentle vortexing and then centrifuged at 735 x g for 1 minute. The labelled oligonucleotide probe was placed onto the top of the column bed and then centrifuged for 2 minutes at 735 x g. The eluent was collected in a 1.5 ml centrifuge tube during the centrifugation.

To determine the percent incorporation of the radioactive nucleotide, the radioactivity (cpm) contained in a 1 µl aliquot of the sephadex-purified probe was compared to the radioactivity contained in a 1 µl aliquot of labelling solution prior to the purification step. The percent incorporation of radioactivity into the probes ranged from 20% to 65%.

Probing of northern blots:

Northern blots were rehydrated in 2x SSC for 5 minutes prior to pre-hybridization. The membrane was then pre-hybridized in pre-hybridization solution (50% v/v formamide, 5x SSPE, 5x Denhardt's solution, 0.1% w/v SDS, 0.1 mg/ml denatured sssDNA; 50x Denhardt's solution = 1% w/v ficoll, 1% w/v polyvinylpyrrolidone, 1% w/v bovine serum albumin) overnight at 43°C. The pre-hybridization solution was removed and hybridization was carried out in pre-hybridization solution containing 1-2 $x10^6$ cpm probe/ml overnight at 43°C. Post-hybridization washes consisted of 2 washes in 2x SSPE (20x SSPE = 3 M sodium chloride, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.4), 0.1% SDS for 15 minutes at room temperature., 2 washes in 0.2x SSPE, 0.1% SDS for 15 minutes at 43°C and rinsed in sufficient changes of 2x SSPE to remove residual SDS. The oligonucleotide probe for collagen α1(I) had an additional 2 washes in 0.2x SSPE, 1% SDS at 45°C prior to rinsing. Probed membranes were wrapped in plastic wrap and exposed to X-omat AR film or Reflections autoradiography film for 1 - 20 days.

Analysis of northern blots:

Autoradiograms were scanned on a Macintosh automated scanner and matched backgrounds were subtracted from each band analyzed. Values were reported as the absolute densitometry reading from the autoradiogram, with the background subtracted, and no compensation were made for variations in radioactivity of the probes or exposure time to the film.

Isolation and growth of ito cells from whole liver:

Two preparations of porcine hepatic stellate cells were used during the course of this thesis research. Initially, hepatic stellate cells were isolated from a 3 cm^3 liver section of a healthy 22 week old female Duroc-Hampshire pig by a modification of the method by Seglen (1972). Liver sections were rinsed in cold Seglen's media, transferred to a sterile petri dish containing cold Seglen's media (145 mM sodium chloride, 67 mM potassium chloride, 10 mM HEPES, pH 7.4) and thin sections were cut using a sterile scalpel. The sections were transferred to a 60 x 15 mm petri dish containing 10 ml collagenase solution (104 units type IV collagenase/ml Seglen's media) at 37°C. The dish was incubated at 37°C for 3 minutes and the liver carefully teased apart using sterile forceps. The collagenase solution containing loosened liver cells was carefully transferred to a 50 ml sterile centrifuge tube placed on ice. Collagenase solution (10 ml) at 37°C was placed on the remaining liver fragments and incubated as above. Digestion and collection of loose liver cells was continued until a final volume of 50 ml collagenase/liver cell suspension was collected (approximately 30 minutes total incubation with the collagenase). This mixture was placed on ice and the large fragments and parenchymal cells were left to settle for 20 minutes. The supernatant (approximately 40 ml) containing parenchymal and non-parenchymal cells was layered over Histopaque™ and centrifuged at 400 x g for 30 minutes. The supernatant layer and buffy layer were collected and re-centrifuged at 250 x g for 10 minutes to pellet the cells. The pellet was resuspended in Dulbecco's modified Eagles medium (DMEM) supplemented with antimycotic/antibiotic (AA) and centrifuged at

250 x g for 10 minutes. Aliquots of the final cell suspension were transferred to a 60 x 15 mm tissue culture dish and allowed to grow in DMEM supplemented with AA, 25% porcine serum and 25% CPSR-1. As the cells grew, the total serum content of the medium was reduced to 20% over a 1 month period.

The second isolate of porcine hepatic stellate cells were from a liver biopsy of a 5 week old female Duroc-Hampshire pig and was isolated as above except that the supernatant collected following collagenase digestion was layered over an equal volume of Optiprep working solution (30% Optiprep working solution = 50% v/v Optiprep, 0.4% sodium chloride, 5 mM tricine, pH 7.8). This was centrifuged at 250 x g for 15 minutes at 4°C and then the cell band interface layer was collected forming a volume of approximately 4 ml. In a fresh 15 ml centrifuge tube 2 ml of 20% Optiprep solution was carefully layered over 2 ml of 25% Optiprep solution. The 4 ml of cell preparation was then carefully layered on top of the 20% solution and then 2 additional layers of 2 ml each 10% and 5% Optiprep were placed over the cell suspension layer. This was centrifuged at 100 x g for 25 minutes at 20°C and hepatic stellate cells were collected from the interphase located between the 5% and 10% Optiprep phases. Isolated hepatic stellate cells were transferred to a 60 x 15 mm tissue culture dish and allowed to grow in DMEM supplemented with AA, 25% porcine serum and 25% CPSR-1. As the cells grew the total serum content of the medium was reduced to 0% porcine serum and 20% CPSR-1 over a 2 month period. The presence of porcine serum was found to arrest the growth of this isolate of hepatic

stellate cells, as did CPSR-1 concentrations below 20%.

These cells exhibited autofluoresence when observed using UV microscopy after 5 days in culture due to retinal stores (Pinzani, 1995). After 2 months in culture the expression of α-smooth muscle actin, a positive indicator for activated hepatic stellate cells (myofibroblasts) (Pinzani, 1995), was confirmed by immunocytochemistry according to the methods of Rowden (1990; 1985). Briefly, cells were subcultured onto microwell slides and allowed to grow for 5 days in DMEM supplemented with AA and 20% CPSR-1. Adhered cells were then rinsed 3 times at room temperature in PBS containing 0.1% saponin. Cells were then fixed in 10% phosphate buffered formalin for 40 minutes followed by 3 more rinses in PBS with 0.1% saponin. The slides were incubated in undiluted horse serum at room temperature for 1 hour followed by a 30 minute incubation in PBS containing murine-derived α-smooth muscle actin antibody diluted 1:1200. This was followed by 3 rinses in PBS containing 0.1% saponin. The slides were incubated for 30 minutes at room temperature in PBS containing a 1:100 dilution of horseradish peroxidase labelled goat anti mouse IgG followed by 3 rinses in PBS with 0.1% saponin. The slides were then counterstained with haematoxylin.

Myofibroblasts were sub-cultured 1:4 upon attaining confluence. Medium was removed from the tissue culture flasks by aspiration and replaced with trypsin/EDTA for 30 seconds. The trypsin solution was removed and fresh solution added onto the

cells. The flask was incubated at 37°C for 5-10 minutes and the digestion was stopped by the addition of 10ml of medium supplemented with AA and serum and/or CPSR-1. The cell suspension was divided among 4 tissue culture flasks and the volume adjusted accordingly with the appropriate medium. The initial myofibroblast culture was maintained in DMEM supplemented with AA, 10% porcine serum and 10% CPSR-1. The second porcine myofibroblast culture was maintained in DMEM supplemented with AA and 20% CPSR-1.

Culture of dermal fibroblasts:

Normal human dermal fibroblasts at passages 9 - 11 were maintained on DMEM supplemented with AA and 10% CPSR-1. Fibroblasts were sub-cultured by the trypsin/EDTA method as described above.

Porcine monocyte-conditioned medium:

Monocyte-conditioned medium (MCM) was prepared as previously described (Peterson and Williams, 1987). Whole blood was diluted 2:3 with RPMI medium and kept on ice until preparation. The diluted blood (8 ml) was layered over 3 ml Histopaque™ in a 15 ml centrifuge tube and centrifuged at 400 x g for 30 minutes at room temperature. The top phase was carefully removed and discarded and the buffy coat layer from 3 tubes was transferred to a fresh centrifuge tube. The cell suspension was diluted to 15 ml with RPMI and centrifuged at 250 x g for 10 minutes at room

temperature. The cell pellet was resuspended in 15 ml of Liebovitz's L15 medium supplemented with CPSR-1 and AA. The cell suspension was transferred to three 15 x 60 mm petri dishes and incubated at 37°C for 24 hours. The media was removed and replaced with fresh L15 media supplemented with 10% CPSR-1 and AA and incubated an additional 24 hours at 37°C for 24 hours. The media was collected, filtered through a 0.22 μm syringe filter unit, aliquoted and stored at -70°C until use. For antibody studies, MCM was pre-incubated with PDGF B/B antibody (1 and 2 μg/ml) at 37°C for 2 hours prior to assay.

Fibroproliferation assay:

Stimulation of cell proliferation was assayed according to the method of Peterson *et al* (1994). Fibroblasts or porcine myofibroblasts were sub-cultured at a density of 8000 cells/well into a 96 well tissue culture plate and incubated for 24 hours at 37°C in DMEM supplemented with AA and 10% CPSR-2, or 20% CPSR-1, respectively. The medium was then replaced with PDGF (0-32 ng/ml), PTX (0-539 μM), metabolite-1 (0-535 μM), n-ethylcarboxyamido adenosine (NECA; 0-561 μM)), or dibutyryl-cAMP (0-0.5 mM) diluted in fresh DMEM supplemented with AA and 10% CPSR-2 or 20% CPSR-1. Following incubation for an additional 22 hours, tritiated thymidine (0.5 μCi/well) was added to each well and the cells incubated a further 2 hours. Cells were harvested onto glass fibre filter paper using a Brandel Cell Harvester (Brandel Laboratories, Maryland). The uptake of tritiated thymidine was measured by liquid scintillation and reported as counts per minute (cpm). Manual cell

counts were performed using a Neubauer type haemacytometer.

Collagen assay:

This method is a modification of that by Diegelmann *et al* (1990). All centrifugations for this assay were done at 400 x g for 10 minutes at room temperature. Myofibroblasts or fibroblasts were plated in 24 well plates at 40000 cells/well and grown to confluence, usually 3 to 7 days, in 1 ml DMEM supplemented with either 10% CPSR-1 (human fibroblasts), 5% pig serum plus 5% CPSR-1 (initial porcine hepatic stellate cell isolate), or 20% CPSR-1 (second porcine hepatic stellate cell isolate). Once confluence was attained, serum replacement on the human fibroblasts was changed to 10% CPSR-2, which has lower mitogenicity, for 24 hours prior to the addition of factors. The media on the porcine fibroblasts was not changed. Factors tested (see below) were diluted in DMEM containing the appropriate serum supplement, 1 μCi/well ^{3}H-proline, and 40 μg/ml ascorbic acid, to a final volume of 0.5 ml/well, and incubated for 24 hours. EDTA (20μl; 0.5 M, pH 8.0) was added to each well, mixed briefly, and the plates were frozen and thawed 3 times. Plates were stored frozen at -70°C until ready for assay. Thawed media was collected into 1.5 ml eppendorf tubes and 50 μl of 100% TCA was added to each tube. The tubes were incubated on ice, or in the refrigerator, for 1 hour and then centrifuged. The supernatant was carefully removed and discarded, the pellet resuspended in 1 ml of 10% TCA, and allowed to precipitate for 1 hour at 4°C. Tubes were centrifuged, the supernatant discarded, and the pellet resuspended in 1 ml cold ethanol:ether (3:1).

After a 1 hour incubation at 4°C the tubes were again centrifuged and the pellet washed a second time in ethanol:ether. After the final centrifugation the pellet was allowed to air dry. The dried pellet was resuspended in 200 µl of collagenase solution (2 mg collagenase/ml in 50 mM tris, pH 7.6, 5 mM $CaCl_2$) and incubated at 37°C for 90 minutes. Non-digested protein was precipitated with the addition of 200 µl of TCA (10%):tannic acid (5%), followed by centrifugation. The supernatant was transferred to a scintillation vial and the pellet resuspended in 200 µl TCA:tannic acid. The samples were centrifuged again and the supernatant pooled with the previous supernatant. The amount of tritiated proline released by collagenase digestion was measured by liquid scintillation and reported as cpm.

The factors tested using this assay were porcine MCM diluted with an equal volume of DMEM supplemented with AA and 20% CPSR-1; porcine MCM pre-incubated with 1 µg/ml or 2 µg/ml anti-PDGF antibody or 1 µg/ml Anti-human factor B antibody for 1 hour at 37°C prior to dilution with an equal part of DMEM supplemented with AA and 20% CPSR-1; 0-64 ng/ml PDGF B/B; 32 ng/ml PDGF B/B pre-incubated for 1 hour with 1 µg/ml anti PDGF antibody; 0-539 µM PTX ± 32 ng/ml PDGF B/B; 0-535 µM M-1 ± 32 ng/ml PDGF B/B; and 0-0.5 mM db-cAMP ± 32 ng/ml PDGF B/B.

Trypan blue exclusion assay:

Trypan Blue stain exclusion was used to test the viability of cultured

myofibroblasts exposed to porcine MCM, PDGF, PTX, M-1, db-cAMP and of cultured fibroblasts to NECA. Cells grown in 24 well plates were exposed to the same conditions as those described for the collagen and fibroproliferation assays. Culture medium was removed and the cells were incubated with trypsin/EDTA for 10 minutes at 37°C. Cells were suspended by gentle tapping of the culture plate and repeated pipetting of each culture. A 100 µl aliquot of the cell suspension was transferred to a 1.5 ml centrifuge tube. Trypan Blue (250 µl, 0.4%) and 150 µl of DMEM supplemented with 10% CPSR-2 was added to the aliquot of cell suspension. The tube was inverted several times to mix the contents and incubated at room temperature for 5 minutes. Cells were counted and viability determined using a Neubauer type haemocytometer.

Statistical analysis:

Statistical analysis was done using a Students' *t* test or a random block analysis of variance (ANOVA). Results were considered significant at $p<0.05$.

Results

Restriction enzyme digestion of plasmid-derived probes:

Probes for human collagen α1(I), α1(III), α1(IV), human collagenase, human β-actin, human TIMP-2 and human G3PDH were cut from their respective plasmids by restriction enzyme digestion as described in the methods section. Probes were separated from the plasmid fragments by agarose gel electrophoresis (fig 2) and the portion containing the probe was cut from the gel (table 3) for use in the hybridization of the northern blots.

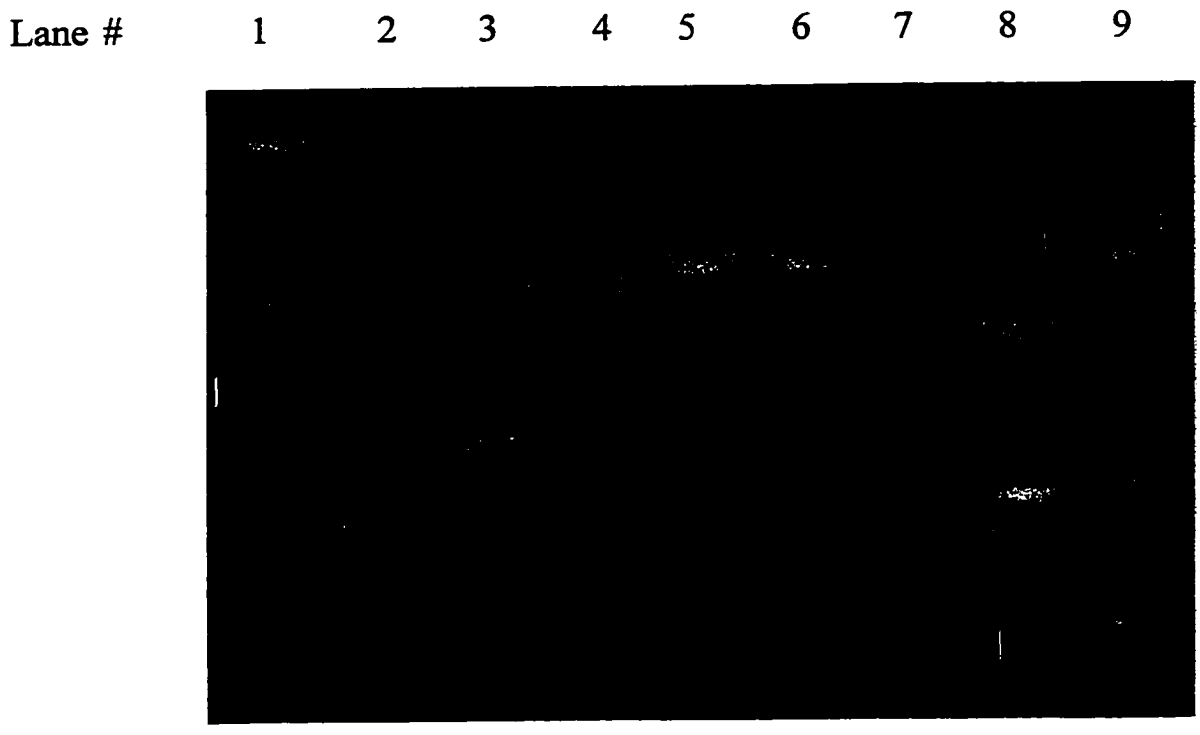

Figure 2. Ethidium bromide stained agarose gel containing restriction enzyme digested plasmids with probe inserts. Lane 1 is the molecular weight marker lambda DNA *Hind III* fragments. Lane 2 is the plasmid containing the collagen α1(I) probe digested with *Eco RI*. Lane 3 is the plasmid containing the collagen α1(III) probe digested with *Aat II* and *Hind III* restriction enzymes. Lane 4 is the collagen α1(IV) containing plasmid digested with *Pst I*. Lanes 5 and 6 are the plasmid containing the collagenase probe digested with *Hind III* and *Sma I*. Lane 7 is the plasmid containing the β-actin probe digested with *Eco RI*. Lane 8 is the plasmid containing the TIMP-2 probe digested with *Pst I*. Lane 9 is the plasmid containing the G3PDH probe disgested with *Pst I* and *Xba I*. See table 2 for sizes of DNA fragments.

Probe	Fragment Sizes (bp)		
Collagen α1(I)	3707	1504	
Collagen α1(III)	3551	1380	
Collagen α1(IV)	3551	1639	
Collagenase	4040	2744	2032
β-Actin	2744	1162	
TIMP-2	2518	898	
G3PDH	3870	938	

Table 3: Molecular weights of plasmid fragments following restriction enzyme digestion and gel electrophoresis (derived from figure 2). The fragment chosen for use as the probe is indicated by the underlined molecular weight.

Collagen mRNA in normal and fibrotic pigs:

Total liver mRNA from 3 control and 4 fibrotic pigs probed for collagen α1(I) produced a doublet banding pattern of 6.5 and 4.8 kb (fig 3). Healthy control pigs had low, but detectable, levels of collagen α1(I) mRNA at 8 and 12 weeks following the start of mineral oil treatment (fig 3; lanes 1, and 4). Little fluctuation in collagen α1(I) mRNA levels was seen between 3 different control pigs from week 8 to 12. Collagen α1(I) mRNA levels were greatly increased in four pigs receiving yellow phosphorus for 8 weeks compared to control animals at the same time point (fig 3; lanes 2 and 3). An additional 4 weeks of yellow phosphorus treatment in two of these pigs showed that the collagen α1(I) mRNA levels remained elevated compared to control animals (fig 3; lane 6).

Blots used for the detection of collagen α1(I) were stripped and probed for collagen α1(III). Total liver RNA samples from healthy control pigs demonstrated a single band of 5.4 kb when probed for collagen α1(III) (fig 3; lanes 1 and 4) whereas RNA from livers of fibrotic animals resulted in a doublet pattern of 5.4 and 4.7 kb (fig 3; lanes 2, 3 and 6). As with collagen α1(I), little fluctuation in the collagen α1(III) mRNA levels were seen in 3 different control pigs or within a control pig from week 8 to 12 (fig 3; lanes 1 and 4). Collagen α1(III) mRNA levels were increased in four fibrotic pigs following 8 weeks of yellow phosphorus treatment when compared to control animals (fig 3; lanes 2 and 3). An additional 4 weeks of yellow phosphorus treatment in two pigs resulted in no further elevation of collagen α1(III) mRNA levels

(fig 3; lane 6). Although both collagens α1(I) and α1(III) mRNAs were increased following 8 and 12 weeks of yellow phosphorus treatment, there was a greater increase in collagen α1(I) mRNA relative to collagen α1(III) mRNA which resulted in an elevation in the ratio of collagen α1(I):α1(III) when compared to control pigs.

Collagen mRNA in fibrotic animals: effect of PTX:

PTX (16 mg/kg) was administered concurrently for 4 weeks to two yellow phosphorus treated pigs following the onset of fibrosis. Fibrosis was verified at 8 weeks by an increase in the collagenous protein content of the liver as assayed by Sirius Red/Fast Green staining (Peterson, 1993). PTX was also administered for 4 weeks to one control pig commencing at 8 weeks. Following 4 weeks of PTX treatment liver mRNA levels for both collagens α1(I) and α1(III) were decreased in fibrotic animals compared to diseased pigs that did not receive PTX during that 4 week period (fig 3; lane 7). This decrease in collagen α1(I) and α1(III) mRNA in PTX treated pigs occurred despite the concurrent administration of yellow phosphorus in this group. Four weeks of PTX treatment in the control pig produced a small decrease in the mRNA levels of collagens α1(I) or α1(III) (fig 3; lane 5) compared to control pigs not receiving PTX (lane 3). Following 4 weeks of PTX treatment the collagen α1(I):α1(III) mRNA ratios in two fibrotic pigs were greatly reduced compared to fibrotic pigs not receiving PTX treatment.

Figure 3. Representative liver collagen collagen α1(I) and α1(III) mRNA levels and ethidium bromide (Eth Br) stain in control (CTL) and fibrotic (FIB) pigs following 8 and 12 weeks of treatment with either mineral oil or yellow phosphorus. Lanes 1 and 4 are from control (CTL) pigs receiving mineral oil for 8 and 12 weeks, respectively; lanes 2 and 3 are from 2 fibrotic (FIB) pigs receiving yellow phosphorus for 8 weeks. Lane 5 is from a control pig which received mineral oil for 12 weeks and pentoxifylline (PTX) concurrently during the 8 to 12 week period. Lanes 6 and 7 are from fibrotic pigs fed yellow phosphorus for 12 weeks, with the latter also receiving pentoxifylline during the 8 to 12 week period. Arrows denote the molecular weights for collagens α1(I) (6.5 and 4.8 kb) and α1(III) (5.4 and 4.8 kb). The apparent increased mobility of the bands in lane 1 was not unique to the mRNA species in that lane as the ribosomal RNA also showed this increase as did the molecular weight marker to the left of that lane (not shown). This procedure was repeated and similar data was obtained from all pig liver sections.

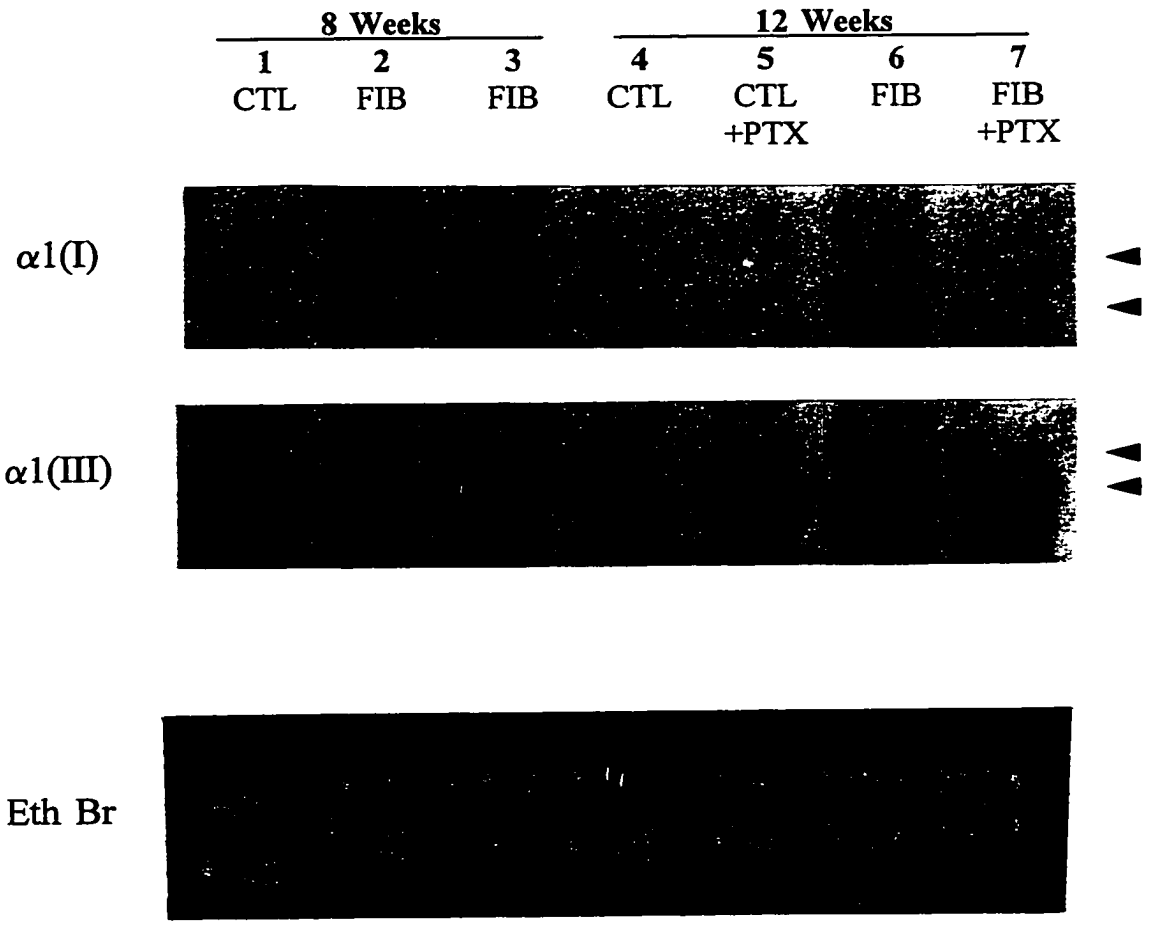

Figure 3: Collagen α1(I) and α1(III) mRNA from porcine livers.

Verification of equal load in northern blots:

A pig liver total mRNA northern blot was probed with β-actin in order to verify equal loading of samples (fig 4), but expression of this housekeeping gene was increased in fibrosis, and decreased in PTX-treated pigs (fig 4). Ethidium bromide staining of the gel showed equal intensities of ribosomal RNA flourescence in all lanes. Therefore, β-Actin was not used further to verify equal loading of RNA samples in northern blots.

Another commonly used housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), was used to verify equal loading of RNA onto the gels. Three attempts with this probe on blots containing total pig liver RNA did not produce any signal suggesting that this human probe does not hybridize with porcine G3PDH. A porcine G3PDH probe was not available. It was, therefore, necessary to rely on spectrophotometric absorption of total RNA samples, as well as the fluorescent intensities of ribosomal RNA bands in the gels following ethidium bromide staining, to ensure the equal loading of total liver porcine RNA samples into the gels.

Collagenase and TIMP-2 mRNA in fibrotic pigs:

Complementary DNA probes for human collagenase or human TIMP-2 were unable to detect mRNA in 3 northern blots containing total pig liver RNA.

Figure 4. Collagen α1(I), β-actin mRNA levels and ethidium bromide (Eth Br) staining from a preliminary northern blot of total pig liver mRNA. Lane 1 is from a fibrotic (Fib) pig receiving yellow phosphorus for 12 weeks. Lane 2 is from a fibrotic pig receiving yellow phosphorus for 12 weeks with 4 weeks of concurrent treatment with pentoxifylline (PTX). Lane 3 is from a fibrotic pig receiving yellow phosphorus for 8 weeks. Lane 4 is from a 16 week fibrotic pig receiving yellow phosphorus for 12 weeks and PTX from weeks 8 to 16. Lane 5 is from a control (Ctl) pig receiving mineral oil for 8 weeks.

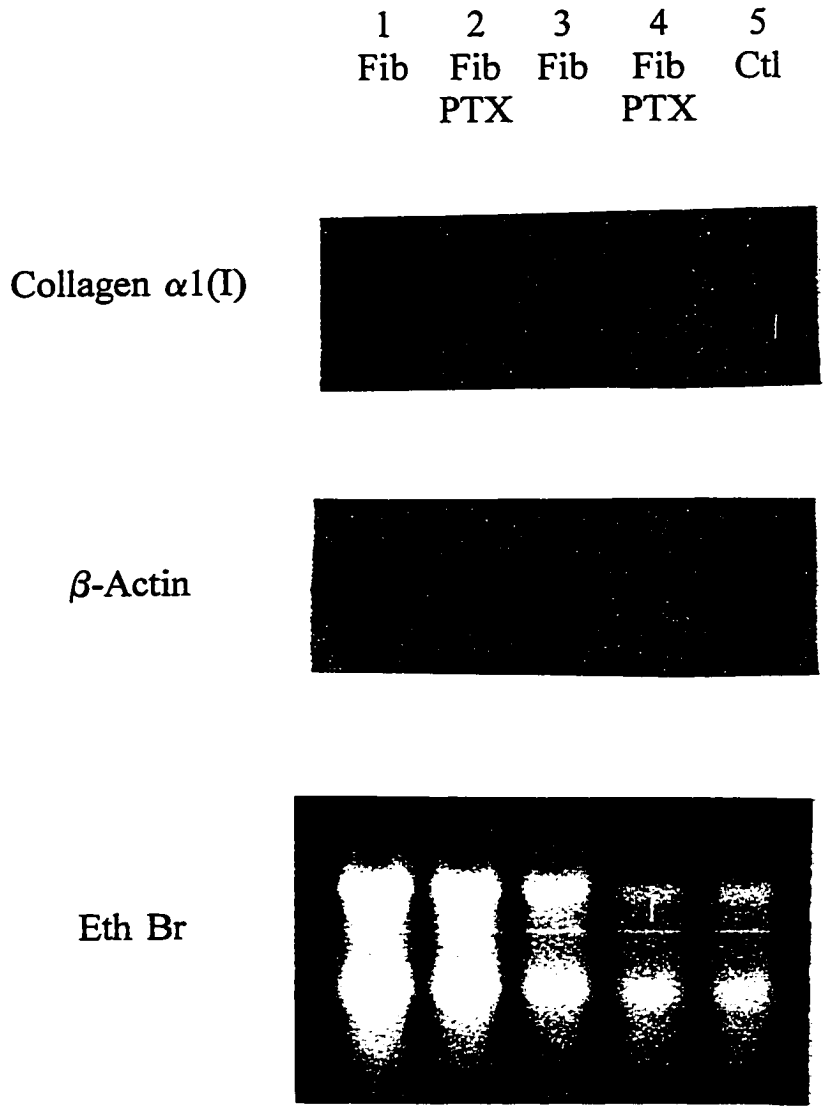

Figure 4: Liver mRNA levels for collagen α1(I) and β-actin in fibrotic and control pigs.

Collagen mRNA in normal, BDL and PTX-treated rats:

Liver collagen α1(I) mRNA was measured in a bile duct-ligated (BDL) rat model of liver fibrosis. Low levels of collagen α1(I) mRNA (6.0 and 4.8 kb) were detectable in 2 control rats (fig 5; lanes 1 and 2), and these levels were elevated in 2 rats which had undergone bile duct ligation (BDL) 4 weeks prior (fig 5; lanes 3 and 4). Treatment of 2 BDL rats for 4 weeks with PTX did not reduce the levels of collagen α1(I) compared to BDL rats not receiving PTX (fig 5; lanes 5 and 6). Probing of northern blots from BDL rats with the collagen α1(III) probe did not produce any detectable mRNA in control or ligated rats in 3 different northern blots. Probing with G3PDH verified equal loading of RNA in the gel (fig 5).

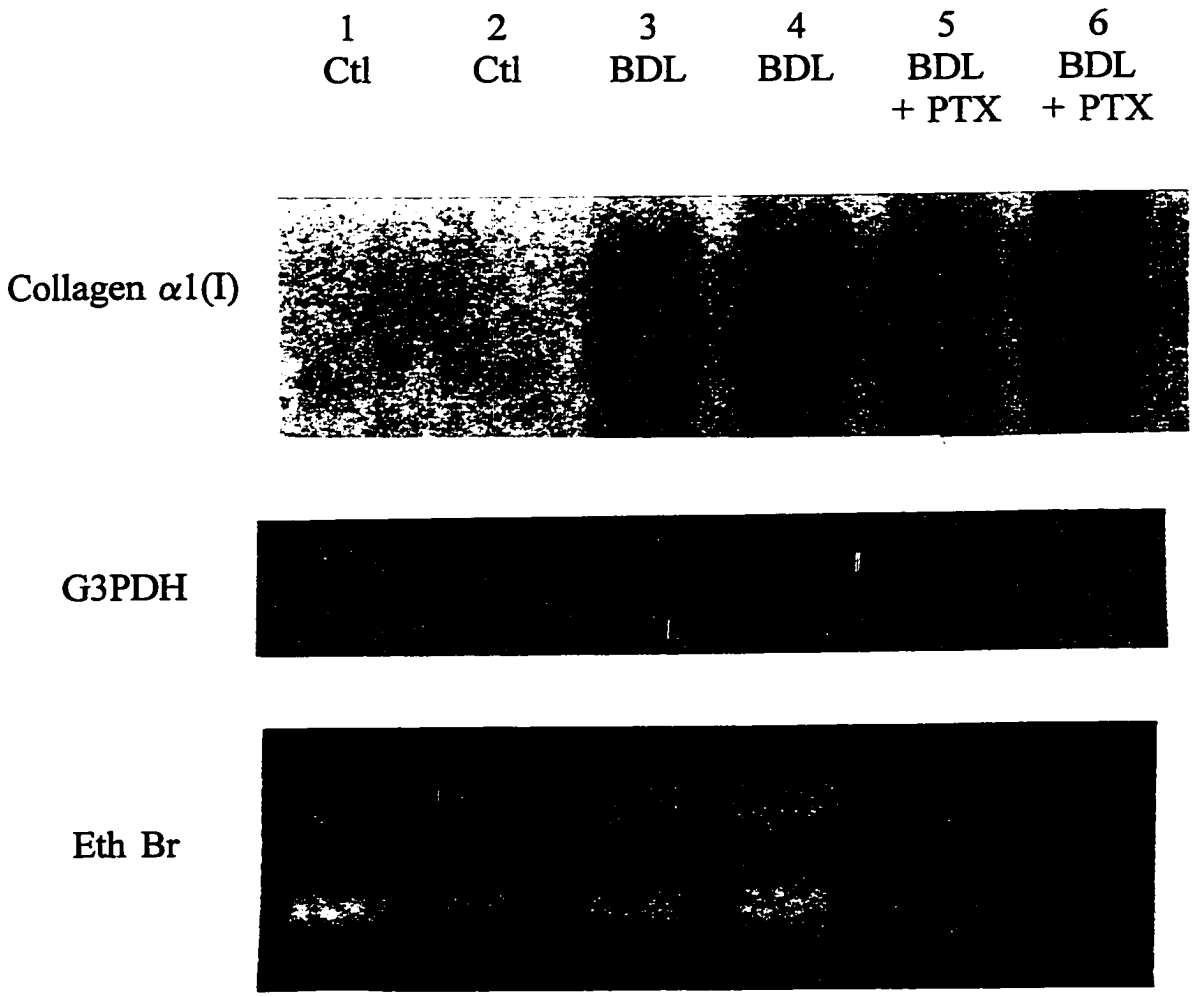

Figure 5. Liver collagen α1(I) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA levels and ethidium bromide (Eth Br) staining from control (Ctl), bile duct ligated (BDL) and pentoxifylline-treated BDL (BDL+PTX) rats. Lanes 1 and 2 are from control rats; lanes 3 and 4 are from rats four weeks after bile duct ligation; lanes 5 and 6 are from bile duct ligated rats receiving PTX during the 4 weeks from ligation to excision of liver.

***In vitro* ^{3}H-proline incorporation: effect of time and β-APN:**

In vitro production of collagen was determined by a modification of the method of Diegelmann *et al* (1990). Collagen production was measured as the release of ^{3}H-proline from collagenase digested proteins produced by confluent cell cultures. To optimize the conditions used, preliminary assays were done examining the incubation time and the effects of different concentrations of β-aminoproprionitrile (β-APN) on collagen production by dermal fibroblasts stimulated with 10% CPSR-1.

Collagen production was measured in confluent cultures of dermal fibroblasts incubated with either 10% CPSR-1 or 0.5% CPSR-2 for 6, 16 and 24 hours. Collagen production by cells stimulated with 10% CPSR-1 was significantly elevated above the levels of collagen produced by those cells incubated with 0.5% CPSR-2 after 24 hours (fig 6). β-APN (50 μg/ml) was used in the original version of this assay (Deigelmann *et al*, 1990) but this compound was found to alter the morphology of the cells as observed by phase contrast microscopy. Cells cultured with β-APN for 24 hours were thinner and appeared to have a reduced cytoplasmic volume such that cell-to-cell contact was reduced in previously confluent cultures. β-APN had no effect on CPSR-1 stimulated collagen production by dermal fibroblasts (fig 7) and was, therefore, not used in subsequent assays.

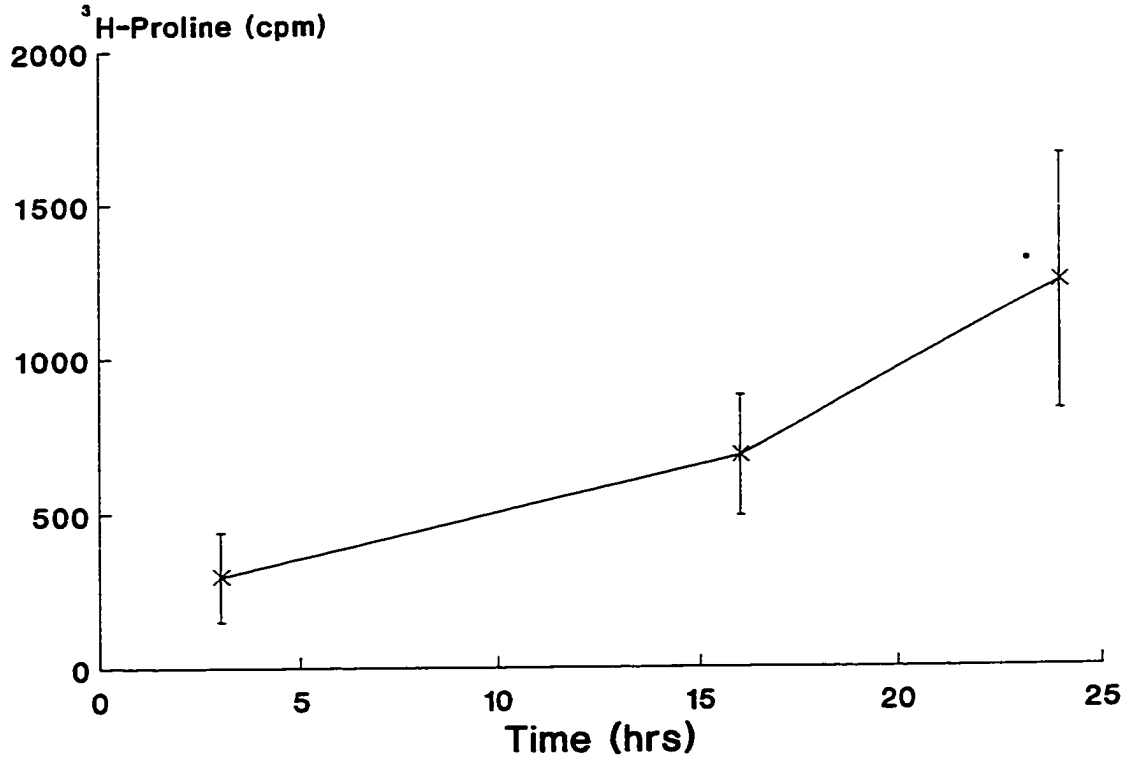

Figure 6: Effect of incubation time on the incorporation of ^{3}H-proline into collagenase sensitive protein by confluent cultures of human dermal fibroblasts. Collagen production was stimulated by the presence of 10% CPSR-1 in the culture medium for the times indicated. Each value represents the average ± S.E.M. for 6 wells.

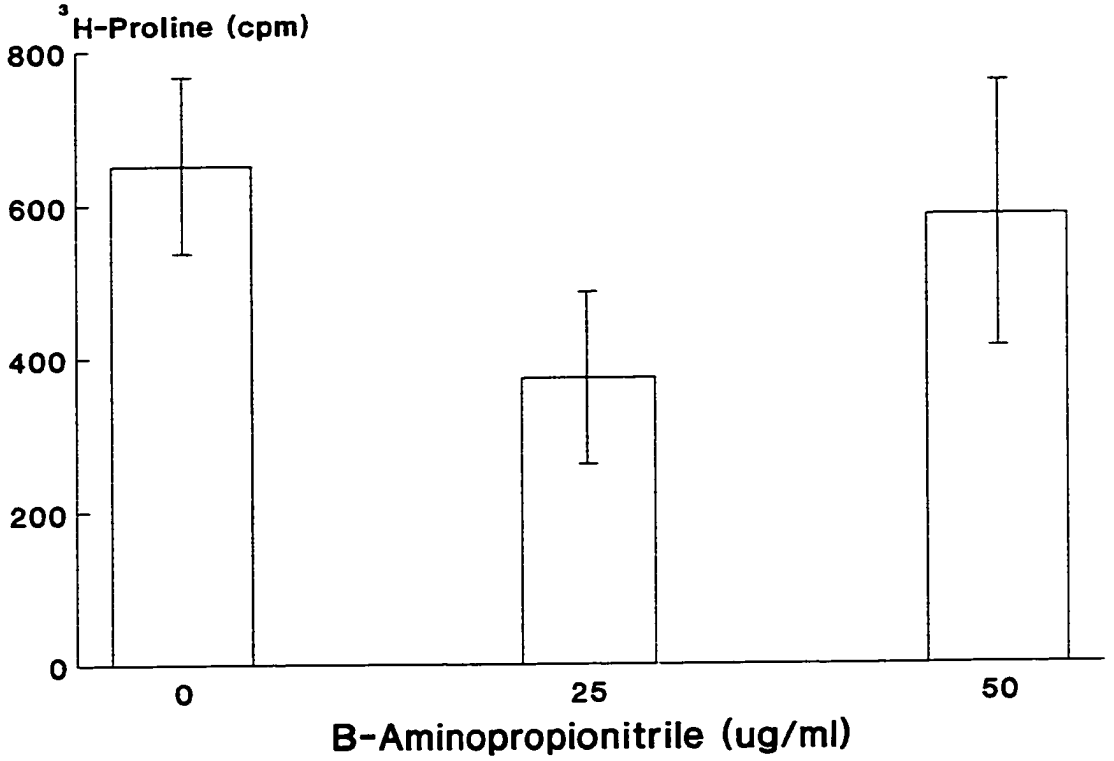

Figure 7: Effect of β-aminoproprionitrile (β-APN) on the production of collagen by confluent cultures of human dermal fibroblasts. Collagen production was assessed by the incorporation of ^{3}H-proline into collagenase-digestible protein. β-APN at these 2 concentrations was found to alter the morphology of the cells. Each bar represents the average ± S.E.M. for 6 wells.

Porcine MCM-stimulated collagen production:

Monocyte-conditioned medium (MCM) was isolated from whole blood obtained from all pigs at 8 weeks following the commencement of mineral oil or yellow phosphorus treatment. MCM was assayed for the ability to stimulate collagen production in confluent porcine myfibroblasts. MCM from 3 control pigs did not stimulate the production of collagens above DMEM alone (fig 8). MCM from 4 fibrotic pigs significantly increased the production of collagenase digestible protein 2.4 times compared to the effect of MCM from control animals (fig 8; $p<0.05$). Cells exposed to control or fibrotic pig MCM showed no difference in cell number (table 4) or viability (table 4) as assayed by trypan blue exclusion compared to cells exposed to DMEM alone.

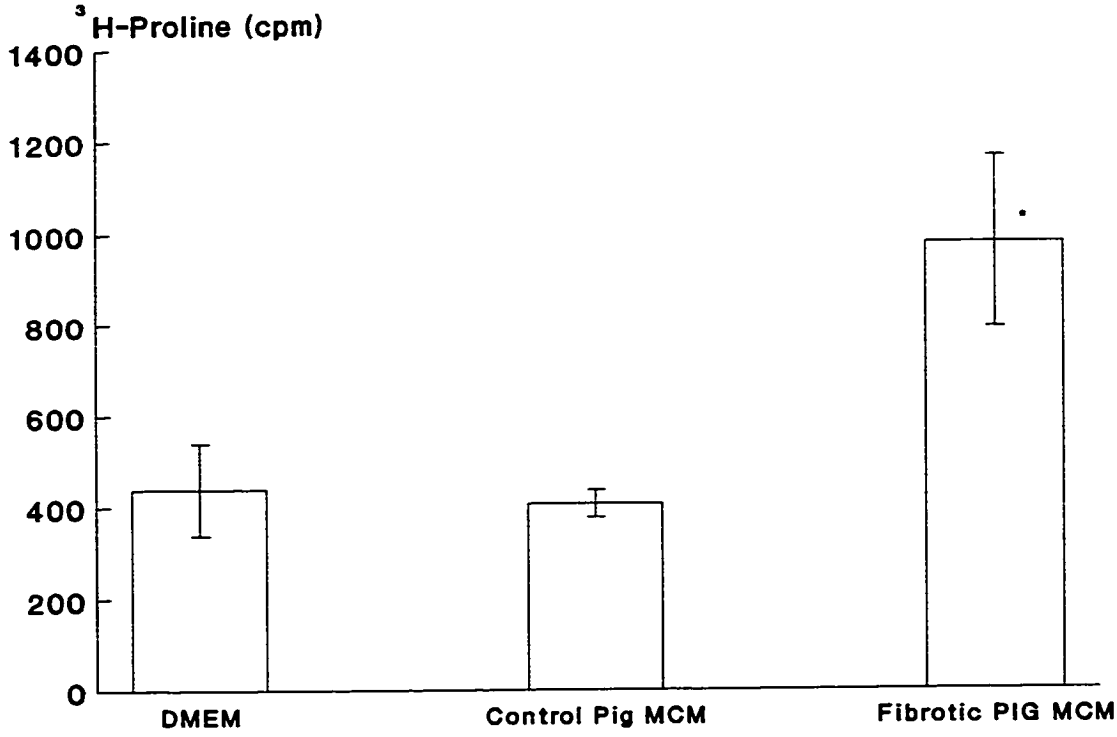

Figure 8. Effects of monocyte-conditioned media (MCM) on collagen production by cultured porcine myofibroblasts. Each pig MCM sample was tested in 6 wells and the results from 3 control pigs and 4 fibrotic pigs were averaged. All samples were derived from animals 8 weeks following the commencement of yellow phosphorus or mineral oil treatment. This experiment was repeated once with similar results. (*$p<0.05$)

	Cell #/well (x 1000)	Viability (%)
DMEM	504 ± 50	98 ± 0.9
Control Pig MCM	515 ± 82	97 ± 1.8
Fibrotic Pig MCM	480 ± 59	97 ± 1.4

Table 4. The effects of MCM on porcine myofibroblast number and viability. Cells were incubated with DMEM alone, MCM from 3 control pigs or MCM from 3 fibrotic pigs. Each value represents the pooled averages from 3 different samples each done in quadruplicate wells. Cell number was done by manual cell count and viability was determined by trypan blue exclusion.

MCM-stimulated collagen production following PTX treatment:

After 8 weeks of yellow phosphorus or mineral oil treatment all 7 pigs continued receiving their regimens but 2 fibrotic and 1 control animal were administered PTX (16 mg/kg/day) concurrently with the yellow phosphorus or mineral oil for 4 additional weeks. MCM was isolated from whole blood obtained from all pigs (at week 12) and tested for the ability to stimulate collagen production in cultured porcine hepatic stellate cells. The MCM from 2 control pigs and one control pig receiving PTX for 4 weeks stimulated collagen production to levels similar to that of cells exposed to DMEM (table 5). The MCM from 2 fibrotic pigs that received 4 weeks of concurrent PTX treatment stimulated collagen production to levels higher than any of the control pig MCM, but did not produce as great a stimulation as did either MCM from 2 fibrotic pigs not receiving PTX (table 5).

	PTX	^{3}H-Proline (cpm ± S.E.M.)
DMEM		2846 ± 519
Mineral Oil	-	2921 ± 119
Mineral Oil	-	2956 ± 435
Mineral Oil	+	2647 ± 294
Yellow Phosphorus	-	7634 ± 855
Yellow Phosphorus	-	8820 ± 519
Yellow Phosphorus	+	4537 ± 531
Yellow Phosphorus	+	4229 ± 246

Table 5. Stimulated collagen production by confluent porcine myofibroblasts exposed to MCM from 12 week mineral oil or yellow phosphorus-treated pigs. Three pigs received concurrent pentoxifylline (PTX) treatment for 4 weeks (+). Collagen production is measured as counts per minute (cpm) of ^{3}H-proline released by collagenase-digested protein. Each value represents the MCM from an individual pig tested in 4 wells.

Effect of anti-PDGF antibody on MCM-stimulated collagen production:

To test for the presence of PDGF activity in MCM from fibrotic pigs, samples were incubated in the presence of anti-PDGF antibody. Pre-incubation of MCM from 4 fibrotic pigs with PDGF B/B antibody (1μg/ml) reduced the net MCM-stimulated collagen production by approximately 45% (fig 9; $p<0.05$). The MCM from 1 fibrotic pig was incubated with a higher concentration of antibody (2 μg/ml) which produced a 46 ± 3% reduction in the net MCM-stimulated collagen production. Anti-human factor B was used for a control and was incubated with the MCM from 1 fibrotic pig. This control antibody did not reduce the ability of the MCM from the fibrotic pig to stimulate collagen production. PDGF B/B antibody (1 μg/ml) was sufficient to neutralize the collagen stimulating effects of PDGF B/B (32 ng/ml) (fig 10). PDGF B/B antibody alone did not affect baseline levels of collagen production (fig 10).

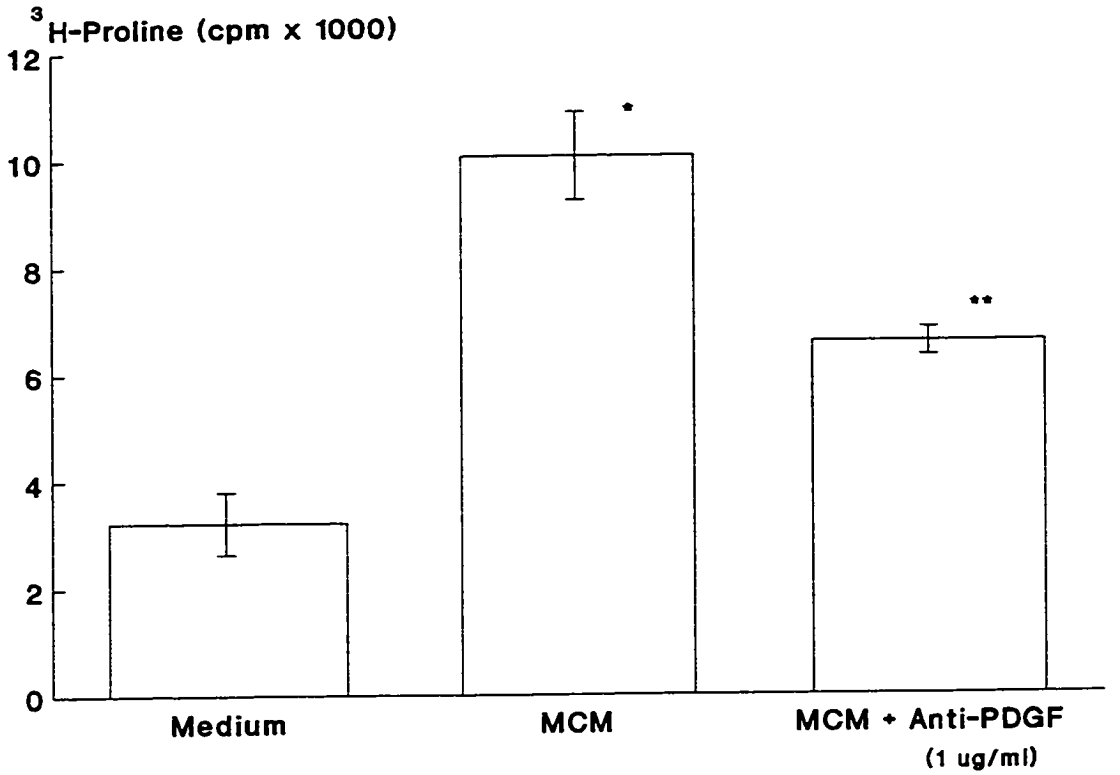

Figure 9. Pre-incubation of fibrotic pig derived MCM with anti-PDGF B/B antibody reduces MCM-induced collagen production by confluent porcine myofibroblasts. Each bar represents the mean ± S.E.M. of 4 different MCM samples, each tested in 6 wells. *significantly different from myofibroblasts not exposed to MCM (Medium) ($p<0.05$). **significantly different from both MCM-stimulated and non-stimulated cells ($p<0.05$).

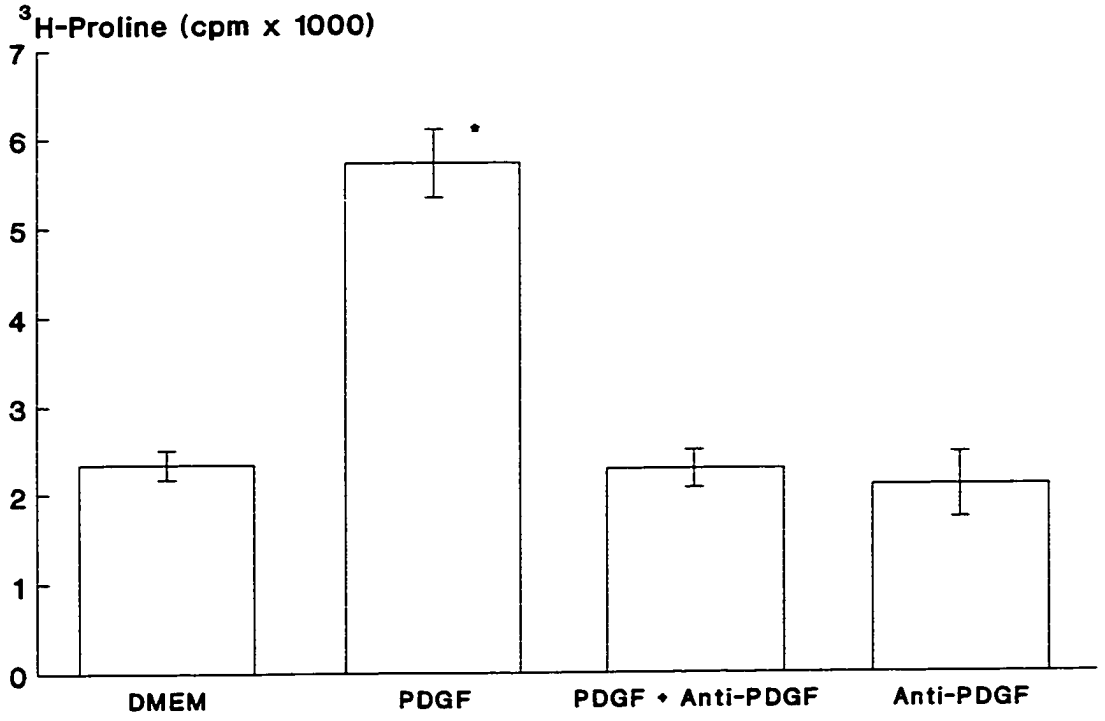

Figure 10. PDGF B/B (32 ng/ml) -stimulated collagen production by confluent porcine myofibroblasts is neutralised by pre-incubation with anti-PDGF B/B. Each bar represents the mean ± S.E.M. of 6 wells (*$p<0.05$).

PDGF B/B Stimulation of Porcine Hepatic Stellate Cells:

PDGF B/B, the major form of PDGF in the pig (Bowen-Pope *et al*, 1989) stimulated the proliferation of non-confluent porcine myofibroblasts as assessed by the uptake of ^{3}H-thymidine (fig 11). PDGF B/B also increased collagen production in confluent porcine myofibroblasts in a dose-dependent manner (fig 12). Maximal stimulation of collagen production occurred with 32 ng/ml PDGF B/B and the EC_{50} was approximately 15 ng/ml. Incubation of confluent porcine myofibroblasts with 32 ng/ml PDGF B/B for 24 hours increased collagen production 250% above control cells but did not increase the cell number as determined by manual cell count (fig 13). There was no difference in cell number between control or PDGF B/B-stimulated cells following an additional 24 hours incubation ($2.95x10^5 \pm 1.06x10^5$ cells/well versus $2.94x10^5 \pm 9.9x10^4$ cells/well, respectively, assayed in 6 wells each).

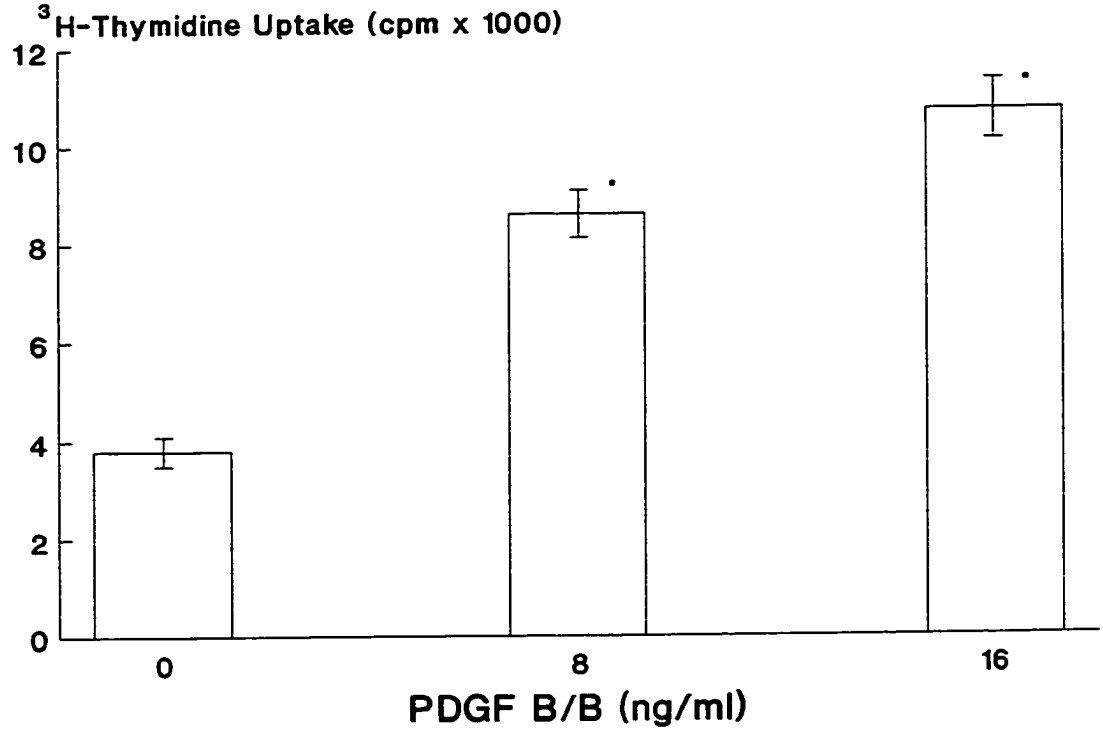

Figure 11. PDGF B/B-stimulated proliferation of non-confluent porcine myofibroblasts as measured by the uptake of ^{3}H-thymidine. Each bar represents the mean ± S.E.M. of 4 wells (*$p<0.05$).

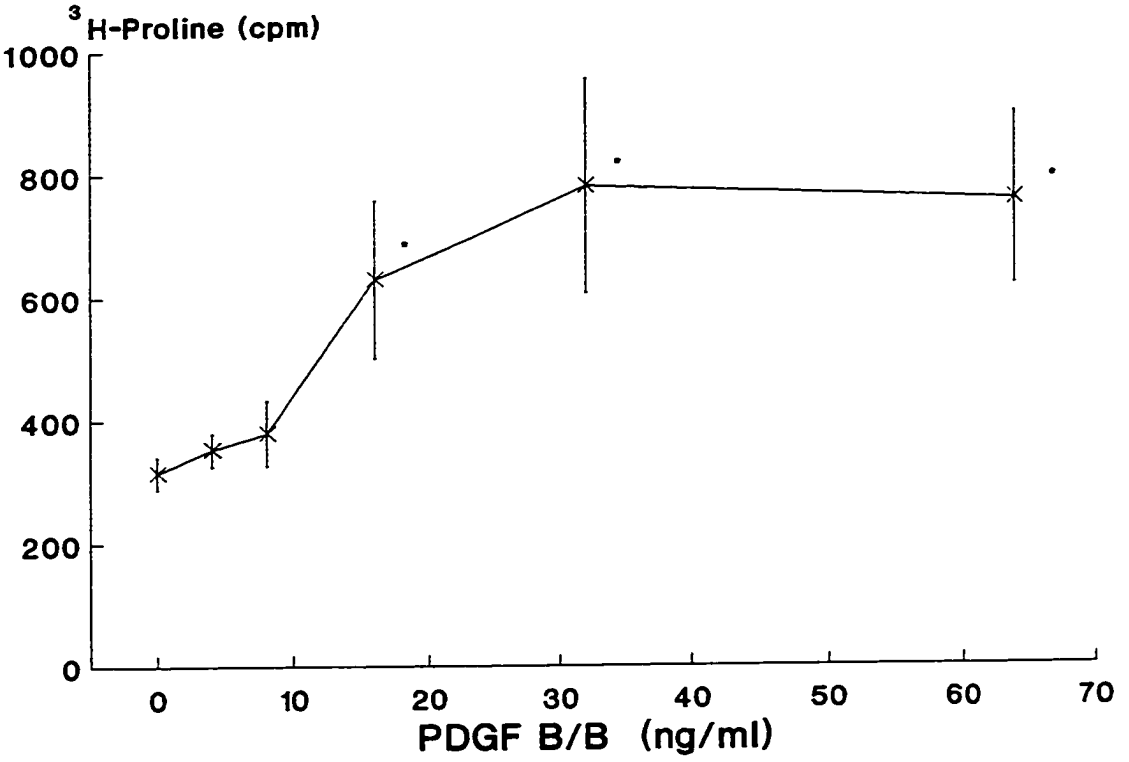

Figure 12. PDGF B/B-stimulated collagen production by confluent porcine myofibroblasts as measured by the release of ^{3}H-proline from collagenase sensitive protein. Each point is the mean ± S.E.M. of 6 wells. EC_{50} is 15 ng/ml PDGF B/B. (*$p<0.05$)

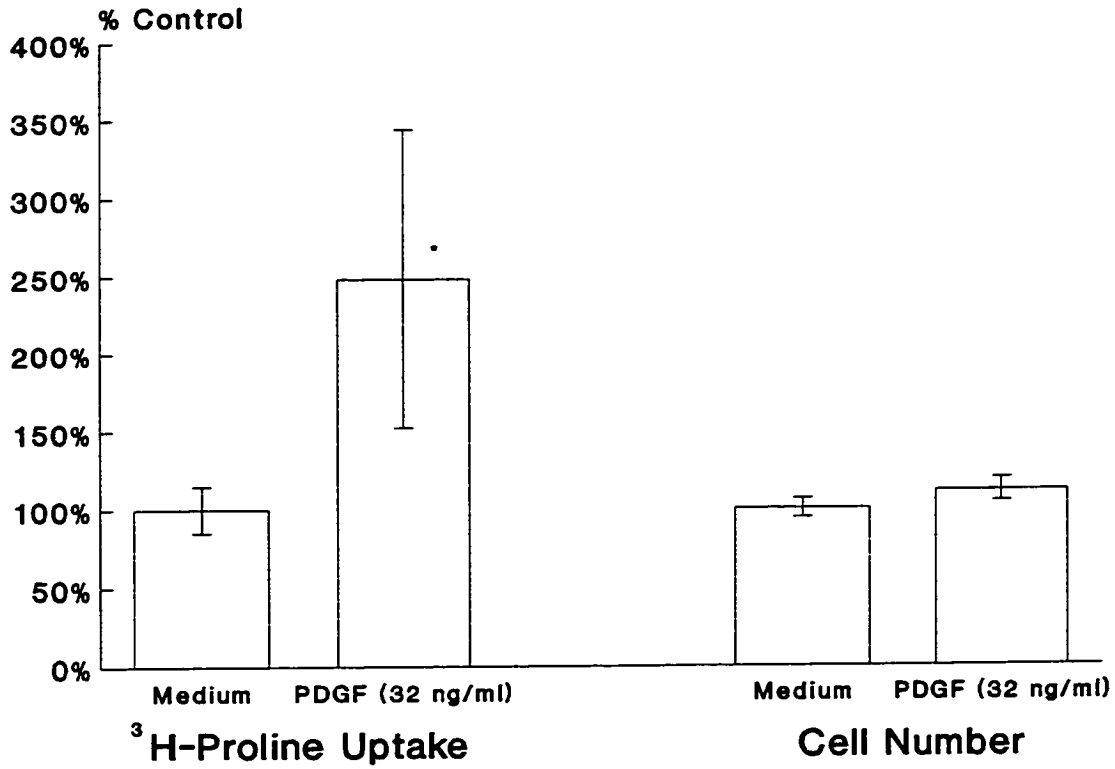

Figure 13. Confluent porcine myofibroblasts incubated with 32 ng/ml PDGF B/B for 24 hours stimulates collagen production but does not induce a concurrent increase in cell number. Each bar represents the mean ± S.E.M. of 6 wells (*$p<0.05$).

PTX inhibition of PDGF-stimulated collagen production:

Confluent porcine myofibroblasts were incubated with PDGF B/B (32 ng/ml) with and without PTX (0 to 539 μM). PTX inhibited PDGF B/B-stimulated collagen production (fig 14) with an IC_{50} of 161 μM. At this concentration PTX inhibited collagen production to levels below those of cells incubated in the presence of DMEM alone. Confluent porcine myofibroblasts incubated with PTX (359 and 539 μM) showed no reduction in cell number (table 6) or in viability (table 6) compared to cells incubated in DMEM as assayed by trypan blue exclusion.

M-1 inhibition of PDGF-stimulated collagen production:

To assess the potential activity of a PTX metabolite to inhibit PDGF-stimulated collagen production, confluent porcine myofibroblasts were incubated with PDGF B/B (32 ng/ml) with and without metabolite-1 (M-1; 0 to 535 μM). M-1 inhibited PDGF-stimulated collagen production in a dose dependent manner and had an IC_{50} of 35 μM (fig 15). At this concentration M-1 inhibited collagen production to levels below those of cells incubated in the presence of DMEM alone. Confluent porcine myofibroblasts incubated with M-1 (178 μM) showed no reduction in cell number (table 6) or in viability (table 6) compared to cells incubated in DMEM as assayed by trypan blue exclusion.

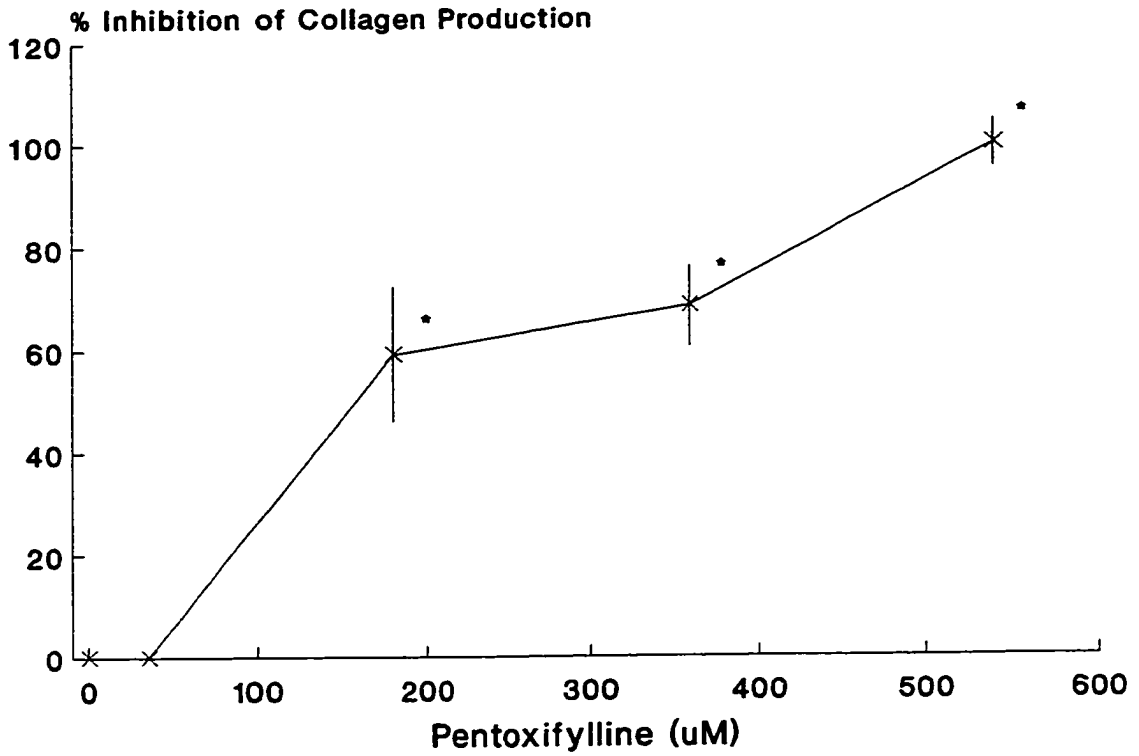

Figure 14. Pentoxifylline inhibits PDGF B/B-stimulated collagen production in confluent porcine myofibroblasts. Cells were incubated with PDGF B/B (32 ng/ml) and varying concentrations of PTX for 24 hours. Collagen production was measured by the incorporation of ^{3}H-proline into collagenase sensitive protein. Each point represents the mean ± S.E.M. of 6 wells. IC_{50} = 161 μM ($^{*}p<0.05$)

	Cell #/well (x 1000)	Viability (%)
DMEM	432 ± 71	98 ± 1.1
PTX (359 μM)	471 ± 50	98 ± 1.9
PTX (539 μM)	475 ± 59	97 ± 1.3
M-1 (178 μM)	465 ± 35	97 ± 1.8
db-cAMP (0.5 mM)	407 ± 116	98 ± 1.4
DMEM	915 ± 100	97 ± 1.2
NECA (187 μM)	1090 ± 60	98 ± 0.9
NECA (374 μM)	1020 ± 62	97 ± 1.6
NECA (561 μM)	960 ± 110	98 ± 1.7

Table 6. Cell number and percent viability of confluent porcine myofibroblasts exposed to PTX, M-1 or db-cAMP for 24 hours, and of confluent human dermal fibroblasts exposed to NECA for 24 hours. Cell number was done by manual cell count and viability was determined by trypan blue exclusion. Each value represents the mean ± S.E.M. of 6 wells (for myofibroblasts) or of 4 wells (for fibroblasts).

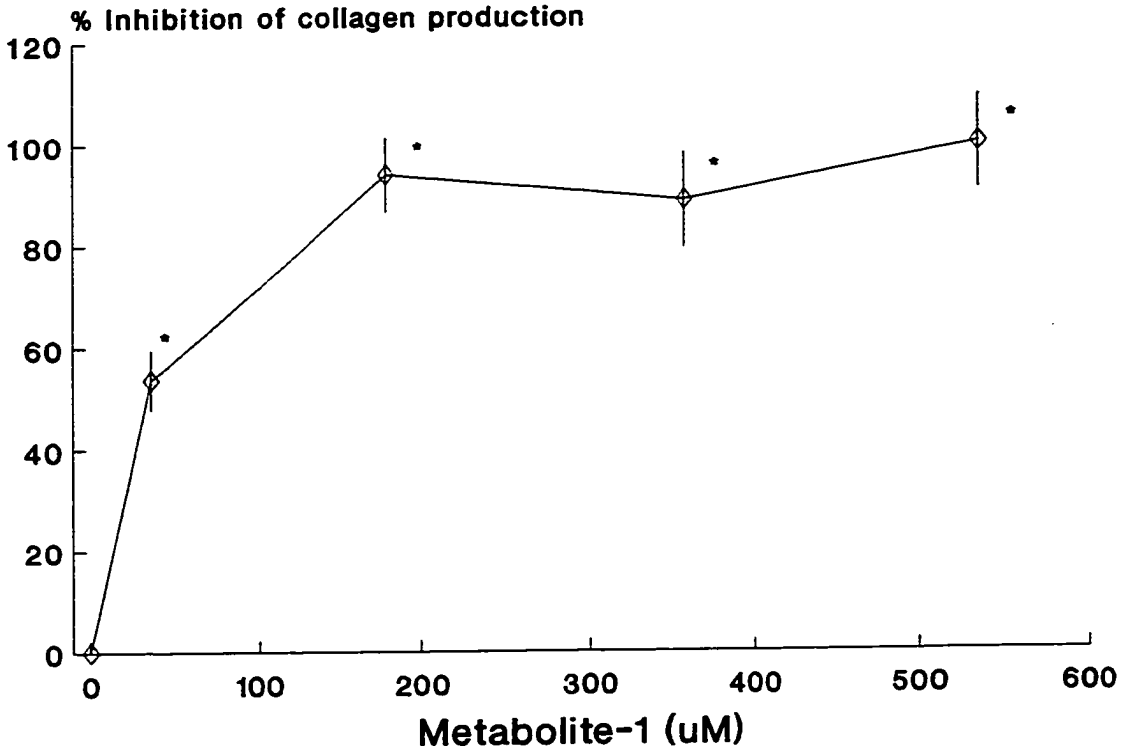

Figure 15. Metabolite-1 of PTX inhibits PDGF B/B (32 ng/ml)-induced collagen production in confluent porcine myofibroblasts. Each point represents the mean ± S.E.M. of 6 wells. IC_{50} = 35 μM ($^{*}p<0.05$)

PDGF + PTX effects on collagen α1(I) mRNA:

Confluent porcine hepatic stellate cells were incubated in the presence of PDGF B/B (32 ng/ml) and/or PTX (539 μM) for 24 hours and the total RNA was isolated from these cells. Northern analysis of collagen α1(I) mRNA using an oligonucleotide probe showed a double banding pattern with molecular weights of 6.5 and 5.0 kb (fig 16). Cells exposed to DMEM produced detectable levels of collagen α1(I) mRNA which were present at the same levels in those cells exposed to PDGF for 24 hours (fig 16; lanes 1 and 2), both PDGF and PTX for 24 hours (fig 16; lane 3), and PTX in the absence of PDGF (fig 16; lane 4). Analysis by densitometry of the autoradiograph and the ethidium bromide fluorescence show that the reduction in the intensity of the collagen α1(I) mRNA band in lane 3 (fig 16) is comparable to the reduction in fluorescence in the same lane.

Figure 16. Collagen α1(I) mRNA levels and ethidium bromide (Eth Br) staining in confluent myofibroblasts exposed to platelet-derived growth factor (PDGF) and/or pentoxifylline (PTX) for 24 hours. Lane 1 is from cells exposed to DMEM supplemented with AA and 20% CPSR-1. Lane 2 is from cells exposed to DMEM supplemented with AA, 20% CPSR-1 and PDGF B/B (32 ng/ml). Lane 3 is from cells exposed to DMEM supplemented with AA, 20% CPSR-1, PDGF B/B (32 ng/ml) and PTX (539 μM). Lane 4 is from cells exposed to DMEM supplemented with AA, 20% CPSR-1 and PTX (539 μM). Collagen mRNA was probed using a 45 bp oligonucleotide as indicated in the methods section. Arrows indicate molecular weight of 6.5 and 5.0 kb.

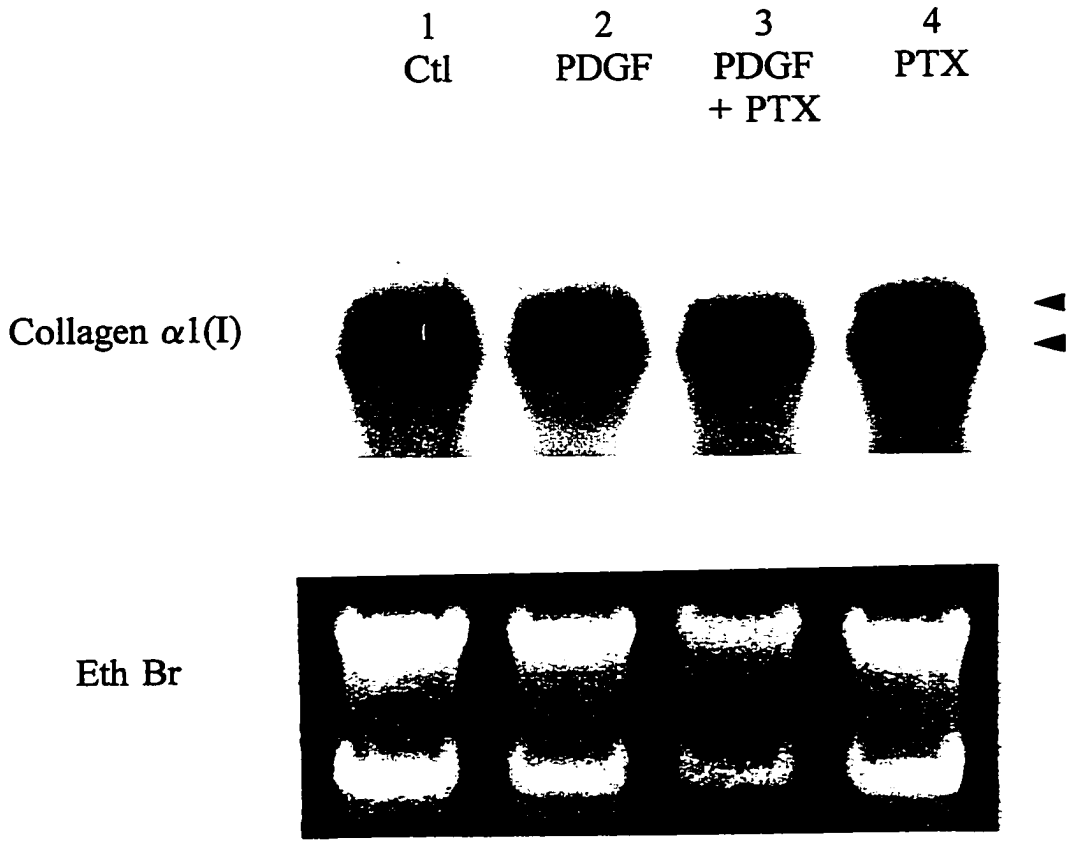

Figure 16: Collagen α1(I) mRNA levels and ethidium bromide (Eth Br) staining of northern blot from confluent porcine myofibroblasts exposed to PDGF and/or PTX for 24 hours.

Effect of NECA on PDGF-stimulated fibroproliferation:

PDGF A/B was previously shown to stimulate the proliferation of human dermal fibroblasts in a concentration manner (Peterson and Isbrucker, 1992; Peterson *et al*, 1994) with maximal stimulation occuring at 8 ng/ml. PTX was also previously shown to inhibit the proliferation of fibroblasts when maximally stimulated with PDGF A/B (Peterson, 1996; Peterson *et al*, 1994; Peterson, 1993) or fetal calf serum (Berman and Duncan, 1990). In order to determine if increased cAMP levels is a mechanism of PTX action, human dermal fibroblasts were stimulated with 8 ng/ml PDGF A/B with and without the A_2 receptor agonist n-ethylcarboxyamido adenosine (NECA). NECA (0-8 μM), a concentration range known to stimulate the production of cAMP in NIH 3T3 fibroblasts (Brackett and Daly, 1994), did not inhibit PDGF-stimulated fibroproliferation in these cells (fig 17). Higher doses of NECA (0-561μM) produced a concentration dependent inhibition of PDGF-induced proliferation (fig 17) although the highest concentration tested (561 μM) did not give maximal inhibition. From the net inhibition, the IC_{50} of NECA was calculated to be 562 μM. Incubation of fibroblasts with NECA (187, 374, 561 μM) for 24 hours did not affect cell number (table 6) and viability (table 6) as assessed by trypan blue exclusion.

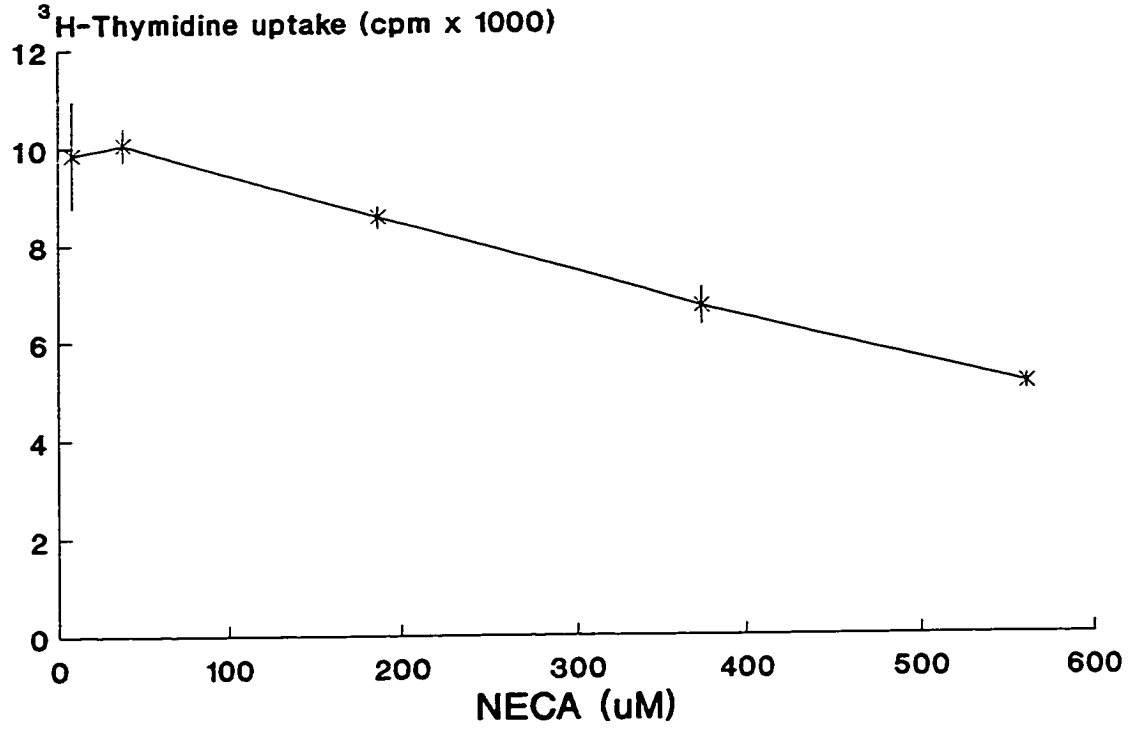

Figure 17. Net inhibition of PDGF A/B induced proliferation of non-confluent human dermal fibroblasts by NECA. Cells were incubated with PDGF A/B (8 ng/ml) and NECA (0 - 8 μM) or NECA (0 - 561μM) for 24 hours. Proliferation was measured by the uptake of ^{3}H-thymidine. Baseline levels were subtracted from levels of cells incubated with PDGF and NECA. Each point represents the mean ± S.E.M. of 4 wells.

Effect of db-cAMP on PDGF-stimulated collagen production and proliferation:

Dibutyryl-cAMP (db-cAMP) was used to simulate the effects of increased intracellular cAMP levels in the porcine myofibroblasts. Maximal stimulation of collagen production by confluent porcine myofibroblasts with 32 ng/ml PDGF B/B was not inhibited by co-incubation of the cells with db-cAMP (0.01-0.5 mM) (fig 18). Proliferation of non-confluent porcine myofibroblasts with 32 ng/ml PDGF B/B was inhibited by this same concentration range of db-cAMP (fig 19). Cell number (table 6) and viability (table 6) were not affected by 24 hour incubation with 0.5 mM db-cAMP.

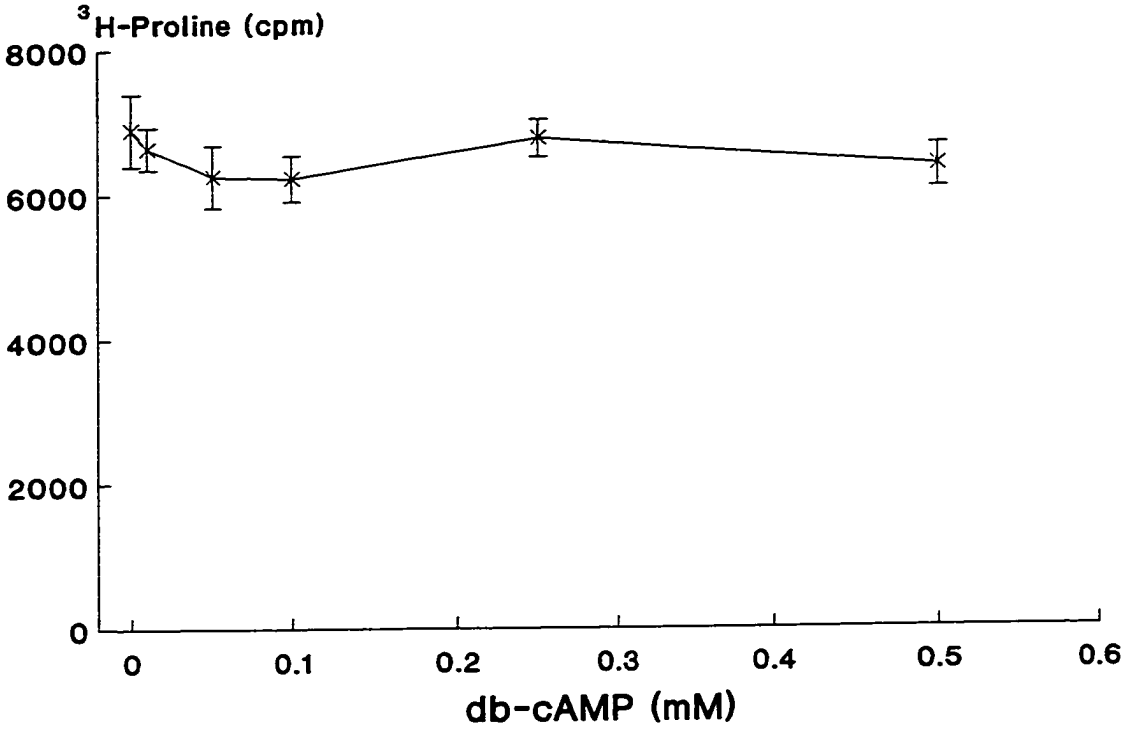

Figure 18. Effect of db-cAMP on PDGF B/B-stimulated collagen production by confluent porcine myofibroblasts. Cells were incubated with PDGF B/B (32 ng/ml) and db-cAMP for 24 hours prior to determination of collagen production. Each point represents the mean ± S.E.M. of 4 wells.

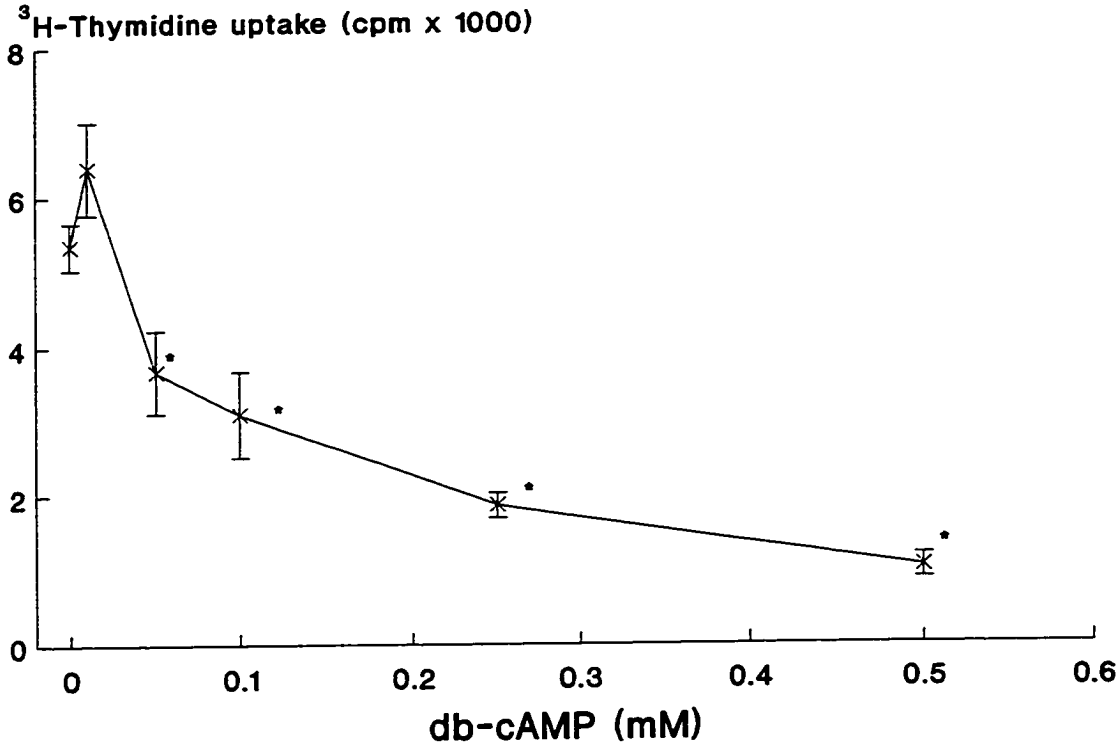

Figure 19. Dibutyryl-cAMP (db-cAMP) inhibition of PDGF B/B stimulated proliferation of non-confluent porcine myofibroblasts. Proliferation was assessed by the uptake of ^{3}H-thymidine following incubation with PDGF B/B (32 ng/ml) and db-cAMP. Each point represents the mean ± S.E.M. of 4 wells (*$p<0.05$).

Discussion

Yellow phosphorus-induced hepatic fibrosis in pigs was previously shown to increase total liver collagen protein levels as assessed by a fast green/sirius red staining method (Peterson and Neumeister, 1996; Peterson, 1993). Liver biopsies and whole blood were obtained from all pigs at various times throughout the treatment period in order to further characterize the mechanism(s) of collagen accumulation in this model. Biopsies were used to examine the fluctuations in total liver mRNA levels for collagens type α1(I) and α1(III) - the most abundant collagen types in the liver (see table 1, Introduction; Pinzani, 1995; Gressner and Bachem, 1994; Friedman, 1993). Monocyte-conditioned medium (MCM), prepared from the whole blood, was used to determine the potential role of immune derived factors in stimulating collagen production.

Liver mRNA levels for collagen types α1(I) and α1(III) were increased in all pigs receiving only yellow phosphorus for 8 and 12 weeks, but not in the control pigs which received mineral oil for this same time period. No further increase in either collagen mRNA levels were observed in pigs receiving 12 weeks of yellow phosphorus versus those receiving only 8 weeks of treatment. The increases in these two collagen mRNA species correlate with the published observations of increased total liver collagen levels in the same fibrotic pigs (Peterson and Neumeister, 1996; Peterson, 1993). Accumulation of collagens within the fibrotic liver of this animal

model is, therefore, due, at least in part, to an increased expression of the genes for these proteins. Other researchers have also found an increase in collagen type α1(I) and/or α1(III) mRNA levels in CCl_4-induced fibrosis in rats (Ikeda *et al*, 1993; Miao *et al*, 1990; Maher and McGuire, 1990), dimethylnitrosamine induced fibrosis in rats (Ala-Kokko *et al*, 1987), bile duct ligated rats (Beno *et al*, 1993; Ikeda *et al*, 1993; Milani *et al*, 1990; Maher and McGuire, 1990), heterologous serum induced fibrosis in rats (Miao *et al*, 1990), and in various forms of liver fibrosis in humans (Greenwel *et al*, 1994; Milani *et al*, 1990b; Clement *et al*, 1986).

The ratio of collagen α1(I):α1(III) mRNA is elevated in fibrotic pigs at 8 and 12 weeks compared to control animals suggesting that the stoichiometry of the extracellular matrix being produced during yellow phosphorus-induced fibrosis is different from that in non-fibrotic tissue. The increase in the ratio of collagen α1(I):α1(III) mRNA is consistent with previous reports from humans with hepatic fibrosis (Greenwel *et al*, 1994; Gressner and Bachem, 1994). This increase in the collagen mRNA ratios is also found in Ito and sinusoidal cells isolated from bile duct ligated or carbon tetrachloride treated rats (Maher and McGuire, 1990). The increase of the collagen α1(I):α1(III) mRNA ratio is indicative of the development of extracellular matrix typical in scar formation (Bissell and Roll, 1990), and demonstrates the presence of active fibrosis in pigs receiving yellow phosphorus for 8 and 12 weeks.

PTX decreases the expression of collagen α1(I) and α1(III) mRNA, as well as the ratio between these two collagen types, after 4 weeks of treatment in 1 control and 2 fibrotic pigs. The decrease in mRNA levels is not due to the removal of the fibrotic stimulus because yellow phosphorus was still being administered during this 4 week period. These results support the histologic and quantitative results reported previously (Peterson and Neumeister, 1996; Peterson, 1993) in which total collagenous protein content in liver biopsies from these pigs was shown to be increased during fibrosis and decreased following PTX treatment. These results also suggest that PTX reduces collagen accumulation in hepatic fibrosis by inhibition of a cellular process that occurs prior to translation of the collagen mRNA. The reduction in the collagen mRNA levels and in the α1(I):α1(III) mRNA ratio in PTX-treated fibrotic pigs implies that the effects of mediators that maintain an active fibrotic process are either being blocked, and/or that fewer mediators are being produced. PTX is reported to decrease the production of TNFα by mononuclear cells (Mattson *et al*, 1996; Kozaki *et al*, 1995; Schandede *et al*, 1992) through transcriptional inhibition of the TNFα gene (Doherty *et al*, 1991). Additional research has shown that PTX is also able to inhibit (a) the phagocytic activity of monocytes (Bessler *et al*, 1986); (b) TGFβ induced collagen production by fibroblasts (Duncan *et al*, 1995); (c) PDGF A/B induced proliferation of fibroblasts (Peterson, 1996; Peterson *et al*, 1994); and (d) PDGF B/B induced collagen production by cultured porcine myofibroblasts (Isbrucker and Peterson, 1995). It is, therefore, likely that the observed effects of PTX on collagen accumulation *in vivo* is the result of the sum of these inhibitory activities of PTX.

It is possible that a decrease in collagenase activity also occurs during fibrosis and that the resultant accumulation of ECM proteins is the summation of increased collagen gene expression and decreased degradation. Interstitial collagenase activity is known to decrease during fibrosis (Murawaki *et al*, 1994; Montfort *et al*, 1990; Perez-Tamayo *et al*, 1987; Maruyama *et al*, 1982), which may be due to increased production of PAI-1 (Arthur, 1994) and/or the increased production of TIMP (Benyon *et al*, 1996; Murawaki *et al*, 1994; Li *et al*, 1993). Complementary DNA probes for human TIMP-2 and collagenase were unable to detect mRNA in northern blots containing pig liver total RNA. The inability of these probes to detect porcine mRNA is possibly due to a poor homology between the human and porcine genes.

Variations in the quantities of total RNA loaded onto gels is another possible reason for the variations in collagen α1(I) and α1(III) levels. Several approaches are used to ensure a minimal amount of sample-to-sample differences in loading. The concentrations of all RNA sample preparations are initially determined by ultraviolet spectrophotometry. This method provides only an approximation of RNA concentration since proteins, DNA and salts can also affect the absorbance values (Maniatis *et al*, 1982). The most commonly used method for ensuring the equal loading of RNA samples is to probe the northern blot for the presence of a ubiquitous and constitutive gene, the expression of which is not influenced by the factors tested. Variations of the examined gene are then corrected for by their relation to the "housekeeping" genes.

Initial analysis of collagen α1(I) and β-actin mRNA from northern blots containing total pig liver RNA showed similar fluctuations in the levels of these two genes. Since the total RNA had been loaded based on spectrophotometric determination of nucleic acid content and since the ethidium bromide fluorescence of the ribosomal RNA bands in each lane were similar, it was concluded that hepatic β-actin expression was affected by fibrosis and PTX treatment. Therefore, β-actin was considered inappropriate for use in the verification of equal RNA loading in northern blots. Hepatic glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA levels are reported to be similar in normal and cirrhotic patients (Gong *et al*, 1996) but are elevated in human hepatic carcinoma cells (Gong *et al*, 1996). The human G3PDH probe was unable to hybridize with porcine RNA following three attempts. G3PDH probes specific to the porcine sequence are not currently available commercially. It was, therefore, necessary to rely on total RNA concentrations and ethidium bromide fluorescence of rRNA bands to ensure that pig liver RNA samples were loaded equally in each lane.

Liver collagen α1(I) mRNA levels were increased in rats following 4 weeks of common bile duct ligation, even with corrections for loading indicated by fluctuations in G3PDH levels. Despite the presence of PTX in one group of bile duct ligated animals this drug was unable to inhibit the progression of fibrosis (Peterson and Neumeister, 1996) or reduce the levels of collagen α1(I) mRNA. Oberti *et al* (1995) also found no reduction in fibrosis, portal venous pressure, or porto-systemic shunts in

4 week bile duct ligated rats treated with PTX. The increase in the collagen α1(I) mRNA levels correlates with the published observation of increased total liver collagen levels in the same bile duct ligated and PTX-treated rats (Peterson and Neumeister, 1996). Other researchers have also found an increase in collagen α1(I) mRNA levels in this model (Beno *et al*, 1993; Ikeda *et al*, 1993; Milani *et al*, 1990; Maher and McGuire, 1990), and lipocytes (myofibroblasts) are considered to be the major source of collagen in this model (Beno *et al*, 1993; Maher and McGuire, 1990).

The fluctuations observed in the rat G3PDH mRNA levels may be due to differences in the quantities of mRNA per lane, but may also indicate that the expression of this enzyme is increased during bile duct ligation induced fibrosis. Factors such as insulin (Alexander *et al*, 1985) and epidermal growth factor (Matrisian *et al*, 1985) are known to increase G3PDH mRNA levels *in vitro*. G3PDH mRNA is also increased in human hepatic carcinoma but not human cirrhotic tissues (Gong *et al*, 1996). These observations indicate that G3PDH is a potentially unreliable housekeeping gene to use as an indicator by which to verify equal mRNA loading since different physiological states can alter its expression. The effects of bile duct ligation on G3PDH levels have not been previously reported.

The differences in effects of PTX on fibrosis in the porcine and rat models is likely due to the differences in the causes and types of fibrosis between these two models rather than a difference in species. In the porcine model the administration of

yellow phosphorus, which causes hepatocyte necrosis (Fernandez and Canizares, 1995), induces an inflammatory response. Inflammatory cytokines, such as TGF-β, are known to increase the expression of collagen genes and the production of collagenous protein (Hansch *et al*, 1995; Brenner *et al*, 1994; King *et al*, 1994). In the bile duct ligated model, there is a physical obstruction which forces the accumulation of bile within the bile ducts and canaliculi. The resultant cholestasis stimulates the proliferation of bile ducts (Tuchweber *et al*, 1996), and although a mild inflammatory event is present in the bile duct ligated model (Scott *et al*, 1994; Milani *et al*, 1990; Kountouras *et al*, 1984), proliferation of bile canaliculi likely occurs independently of the inflammatory event. Because this cholestatic model is due to a mechanical injury and functional blockage of the bile drainage system it is not surprising that PTX is unable to inhibit the state of fibrosis. Also, if the anti-fibrotic properties of PTX observed in the pig model are due to an inhibitory action of this drug against fibrogenic cytokines (Peterson and Neumeister, 1996), then PTX would not be expected to work in this model since MCM from bile duct ligated rats is not fibroproliferative (Peterson and Neumeister, 1996).

Confluent monolayers of activated hepatic stellate cells (myofibroblasts), the primary collagen producing cells of the liver (Pinzani, 1995; Gressner and Bachem, 1994; Friedman, 1993; Ramadori, 1991; Maher and MCGuire, 1990), were used to investigate the mechanisms by which collagen production is altered in fibrosis and how PTX reduces the accumulation of collagen during fibrosis. Although TGFβ, a

monocyte-derived cytokine (Decker, 1990), is known to be a potent stimulator of collagen synthesis (Hansch *et al*, 1995; Brenner *et al*, 1994; King *et al*, 1994), there remains the question as to whether PDGF has a role in promoting the accumulation of collagens during fibrosis. PDGF was previously shown to be a monocyte-derived cytokine from patients with liver disease that is a potent stimulator of fibroblast proliferation (Peterson and Isbrucker, 1992). PDGF is also known to stimulate the proliferation of other cells of mesenchymal origin such as glial cells (Westermark and Wasterson, 1976), mesangial cells (Choudhury *et al*, 1993), and hepatocyte myofibroblasts (Pinzani *et al*, 1994). It is possible that the increased PDGF levels that occur during fibrosis promote the accumulation of collagens by increasing the number of collagen-producing cells rather than directly affecting the gene expression for these ECM proteins. In this regard, the increased levels of collagens α1(I) and α1(III) mRNA observed in the pig liver may be a reflection of an increased number and population density of hepatic stellate cells rather than an increased expression of these genes. It is, therefore, important to separate the events of proliferation and increased collagen production in order to determine the extent of the influences of PDGF during fibrosis. To minimise the effects of proliferation on collagen production *in vitro*, these experiments used confluent cultures of myofibroblasts to promote the phenomenon of contact-inhibition of cell growth. Since PDGF increases actual cell number, as well as the uptake of ^{3}H-thymidine, within 24 hours in non-confluent cultures (Peterson *et al*, 1994) collagen production in confluent cultures was compared to changes in cell number under the same conditions.

MCM from patients with liver disease (Peterson and Isbrucker, 1992) and from yellow phosphorus treated pigs (Peterson and Neumeister, 1996) have previously been shown to stimulate the proliferation of dermal fibroblasts *in vitro* while samples from bile duct ligated rats (Peterson and Neumeister, 1996) and heterologous serum-induced fibrosis in rats (Hodgson and Peterson, 1995) did not. Exposure of primary cultures of rat hepatic stellate cells to rat Kupffer cell-conditioned medium activates the stellate cells, induces the expression of PDGF receptors, and promotes their proliferation (Friedman and Arthur, 1989). In order to assess whether monocyte-derived cytokines can also affect extracellular matrix synthesis, the MCM from fibrotic and control pigs was assayed for its ability to stimulate collagen production by cultured porcine myofibroblasts. The MCM from 4 pigs that received yellow phosphorus for 8 weeks significantly increased the collagen synthesis by confluent myofibroblasts compared to the MCM from 3 control pigs. This same MCM from the fibrotic pigs did not increase the cell number in the culture system. These results indicate that peripheral circulating monocytes from diseased pigs are actively secreting factor(s) which are fibrogenic to density-arrested cultured myofibroblasts and demonstrates that increased collagen production can occur in the absence of proliferation.

All 4 fibrotic pigs continued receiving yellow phosphorus from weeks 8 to 12, but 2 pigs were also administered PTX concurrently during this 4 week period. The MCM from both pigs receiving the yellow phosphorus alone for 12 weeks stimulated collagen production to levels similar to that found after 8 weeks of yellow phosphorus

administration. The MCM from the 2 fibrotic pigs receiving PTX also stimulated the production of collagen by cultured myofibroblasts, but to levels which appear higher than those of control pigs and lower than those of the fibrotic pigs not receiving PTX. Therefore, PTX treatment reduces the amount of fibrotic mediators being produced in the pigs administered yellow phosphorus. These results are in agreement with those of others where PTX was shown to inhibit the release of TNFα from monocytes (Mattson *et al*, 1996; Schandene *et al*, 1992) and Kupffer cells (Kozaki *et al*, 1995).

Anti-PDGF antibody was previously reported to inhibit the proliferative ability of MCM derived from liver disease patients (Peterson and Isbrucker, 1992). Pre-incubation of the MCM from 4 fibrotic pigs with PDGF B/B antibody (1 μg/ml) inhibited the ability of that MCM to stimulate collagen production by 50%, indicating that PDGF is one monocyte-derived cytokine that is important in regulating collagen synthesis and suggests a role for PDGF in signalling the accumulation of collagens during active hepatic fibrosis. Higher concentrations of PDGF antibody did not produce a further inhibition of the collagen-stimulating ability of the MCM. This demonstrates that other collagen stimulating factors, such as TGFβ or IL-1, are likely also present in the MCM from fibrotic pigs. However, these results do not indicate whether PDGF alone is able to stimulate collagen production, or whether it potentiates the effects of other collagen-inducing factors present in MCM.

PDGF stimulates the proliferation of fibroblasts (Peterson, 1996; Lepisto *et al*,

1995; Peterson *et al*, 1994) and there is an increased expression of PDGF receptors on activated hepatic stellate cells during fibrosis (Pinzani, 1995; Gressner and Bachem, 1994; Friedman, 1993; Heldin *et al*, 1991). PDGF is shown here to stimulate the proliferation of non-confluent porcine myofibroblasts as is reported by others (Davis *et al*, 1993; Davis *et al*, 1991; Pinzani *et al*, 1989). PDGF B/B is also shown here to stimulate the production of collagen in confluent porcine myofibroblasts in a concentration-dependant manner without a subsequent increase in cell number. Maximal collagen production by porcine myofibroblasts was attained by 32 ng/ml PDGF B/B, four times higher than the reported concentration necessary for maximal stimulation of dermal fibroblast proliferation with PDGF A/B (Peterson *et al*, 1994). This duplicity of stimulatory effects of PDGF demonstrates the importance of this cytokine in fibrogenic events and makes PDGF a prime target for therapeutic intervention of liver disease.

Cultured porcine myofibroblasts show similar levels of collagen α1(I) mRNA whether the cells are exposed to basal medium or PDGF B/B for 24 hours. Similar culture conditions clearly show that PDGF induces the production of collagens by these cells within 24 hours as assessed by the incorporation of ^{3}H-proline into collagenase-sensitive protein. Although the possibility remains that collagen α1(I) mRNA is elevated and returns to basal levels by 24 hours, other researchers clearly show that TGFβ-induced increases in collagen α1(I) mRNA are still elevated at this time (Houglum *et al*, 1995). The reported effects of PDGF on collagen production are

contradictory (Yi *et al*, 1996; Lepisto *et al*, 1995; Tan *et al*, 1995; Owens and Milligan, 1994; Pierce *et al*, 1994). These inconsistent reports are likely due to variations in cell types, culture conditions and the method of assessing collagen production. In our culture conditions PDGF B/B was able to induce a significant elevation of collagenase-sensitive protein production following 24 hours exposure, but similar conditions did not appear to alter the level of collagen α1(I) gene expression. It is possible that PDGF is inducing non-α1(I) collagen gene expression since Hansch *et al* (1995) showed that PDGF increases α1(IV) collagen mRNA levels in mesangial cells. It is also possible that PDGF affects the efficiency of the post-translational processing of collagens. Both prolyl hydroxylase and protein disulphide isomerase activites are known to be mediated by cytokines (Kawaguchi *et al*, 1992), and PDGF also increases amino acid uptake into cells (Owen *et al*, 1982).

PTX is known to inhibit PDGF A/B-induced proliferation of fibroblasts (Peterson, 1996; Peterson *et al*, 1994) and inhibit the progression of fibrosis in yellow phosphorus treated pigs (Peterson, 1993). It is reported here that PTX also inhibits PDGF B/B-induced collagen production in porcine hepatic stellate cells and has an IC_{50} of 161 μM. This IC_{50} is 25% higher than that reported for its inhibitory effects on PDGF A/B-induced proliferation of dermal fibroblasts (Peterson *et al*, 1994). A concentration of 161 μM PTX reduces the proliferative response of fibroblasts to PDGF by approximately 80% (Peterson *et al*, 1994). Trypan blue exclusion assays and cell counts of confluent cultures of myofibroblasts exposed to PTX verified that

the inhibitory effects of this drug on PDGF B/B-stimulated collagen production were not due to decreased viability or cell numbers.

PTX had no effect on collagen α1(I) mRNA levels in myofibroblasts either in the presence or absence of PDGF. Duncan *et al* (1995) demonstrated that PTX decreases α1(I) mRNA levels in dermal fibroblasts stimulated with serum and that this inhibitory effect required the synthesis of new protein. The discrepancy between these results is possibly due to the differences in collagen stimulating factors used. In our experimental system myofibroblasts are grown in the presence of a controlled process serum replacement with PDGF B/B added as the collagen-inducing factor. In the assay used by Duncan *et al*, (1995), dermal fibroblasts were stimulated to produce collagen by the addition of fetal calf serum, a supplement which possible contains several different collagen inducing stimuli, such as TGFβ or IL-1. From our results it appears that the PTX-mediated down regulation of PDGF-induced collagen production does not occur by directly decreasing the collagen α1(I) gene expression, but rather occurs either at a post-translational step in the processing of collagen, or by inhibiting the signal transduction of PDGF.

Metabolism of PTX *in vivo* results in the formation of seven metabolites (Samlaska and Winfield, 1994; Ward and Clissold, 1987). Metabolite-1 (M-1), which is produced by the reduction of PTX at the N-1 position (Ward and Clissold, 1987), inhibits PDGF-induced fibroproliferation (Peterson, 1996) and is active in the

treatment of peripheral circulatory disorders (Ward and Clissold, 1987). M-1 inhibits PDGF-induced collagen production by cultured porcine myofibroblasts and has an IC_{50} of approximately 35 μM; a concentration that is less than one quarter of the IC_{50} for PTX, and less than one sixth of that reported for the inhibition of PDGF-induced fibroproliferation by M-1 (Peterson, 1996). This result indicates that the observed *in vivo* effects of PTX on collagen accumulation in liver fibrosis is largely mediated by the active metabolite of PTX rather than the parent compound itself.

From these results it can be concluded that the decrease in collagen α1(I) and α1(III) mRNA levels in PTX-treated fibrotic pigs is due to both the reduced levels of fibrogenic factors secreted by monocytes following PTX treatment and also by the direct inhibition of PDGF-stimulated collagen production by PTX and its metabolite. The mechanism by which PTX inhibits the actions of PDGF is not known. Prior results from this laboratory suggest an interference by PTX in the post-receptor signalling of PDGF since PTX does not inhibit the binding of PDGF to PDGF receptors (Slysz and Peterson, 1994) nor does PTX act on either the A_1 or A_2 adenosine receptors (Peterson, 1996). PTX is a non-selective inhibitor of cAMP phosphodiesterases (Meskini *et al*, 1994) and has been shown to increase the intracellular cAMP levels in cultured cells (Sinha *et al*, 1995; Yonemaru *et al*, 1991; Bessler *et al*, 1986). Both fibronectin (Coats and Brecher, 1993; Miao *et al*, 1993) and collagen (Yamamoto *et al*, 1994; Andrabi *et al*, 1992; Perr *et al*, 1989) production are inhibited by elevations in cAMP. Also, elevations in cAMP can block PDGF

signal transduction by inducing the dephosphorylation of the activated PDGF receptor (Böhmer and Böhmer, 1996) and by inhibiting Ras/Raf interactions in the MAP kinase cascade (Cook and McCormick, 1993).

NECA (0.25 - 8 μM), an adenosine A_2 agonist (Dalziel and Westfall, 1994) did not inhibit PDGF A/B-stimulated proliferation of fibroblasts at a concentration range which is known to stimulate maximal production of cAMP in NIH 3T3 fibroblasts (Brackett and Daly, 1994) and rat Kupffer cells (Reinstein *et al*, 1994). Higher concentrations (>200 μM) of NECA did inhibit PDGF-stimulated proliferation of the fibroblasts, although maximal inhibition was not attained at 560 μM, the highest concentration tested. From the net inhibition, the IC_{50} of NECA was calculated to be greater than the highest concentration used. Although the more commonly used concentration ranges for NECA *in vitro* are generally less than 50 μM, there was no loss of viability when cells were incubated with 560 μM NECA for 24 hours. From these results it is unlikely that the inhibitory actions of NECA on PDGF-stimulated fibroblast proliferation is mediated by elevations in intracellular cAMP.

To directly assess the effect of increased intracellular cAMP levels on PDGF-stimulated proliferation and collagen synthesis in myofibroblasts, db-cAMP was used. Dibutyryl-cAMP (0-0.5 mM) showed a concentration-dependent inhibition of PDGF B/B-stimulated proliferation and had an IC_{50} of 0.2 mM. Reinstein *et al* (1994) demonstrated that 1 mM PTX induced the production of cAMP in Kupffer cells to a

level of 390 fmol/10^6 cells; an amount of cAMP which is 10^8 times lower than used here in the form of db-cAMP. A lower concentration range of db-cAMP (0 - 10 μM) showed no effect on the PDGF B/B-induced proliferation of hepatic stellate cells.

In contrast to its effects on proliferation, db-cAMP (0-0.5 mM) did not inhibit PDGF B/B-induced production of collagen by confluent porcine hepatic stellate cells. Therefore, PTX-inhibition of PDGF-induced collagen production is not mediated by the intracellular elevation of cAMP. These results also suggest that the PDGF signal transduction pathway leading to cellular proliferation is not the same as that PDGF signal transduction pathway leading to increased collagen production. It has recently been reported that cAMP mediated down regulation of MAP kinase activity may not be universal in all cell types: EAhy 926 endothelial cells (McLees *et al*, 1995) and PC-12 neuronal cells (Vaillancourt *et al*, 1994) showed no inhibition of stimulated MAP kinase activity by agents that induce or mimic cAMP. Therefore, PTX may raise cAMP levels in hepatic stellate cells, but its effects on PDGF-induced proliferation or collagen production occur via a signal transduction mechanism that is not affected by cAMP.

Conclusions

Several conclusions can be drawn from the *in vivo* and *in vitro* data presented in this thesis:

1. The increase in liver collagenous protein in the porcine model of fibrosis is due, at least in part, to the increase in collagen α1(I) and α1(III) gene expression.

2. The reduced levels of liver collagenous protein in fibrotic pigs treated with PTX is due, at least in part, to the decrease in the expression of the collagen α1(I) and α1(III) genes.

3. Collagen α1(I) mRNA is also elevated in the rat bile duct ligated model of fibrosis, but that PTX does not affect the level of collagen α1(I) gene expression in this animal model.

4. MCM from fibrotic pigs stimulates the production of collagen by cultured porcine myofibroblasts above baseline levels.

5. Administration of PTX to fibrotic pigs reduces the collagen-stimulatory properties of their MCM.

6. PDGF accounts for approximately 50% of the collagen stimulatory quality of MCM from fibrotic pigs.

7. PDGF can stimulate both the proliferation and production of collagens by cultured porcine myofibroblasts, and that collagen production occurs in the absence of proliferation.

8. PTX and its metabolite, M-1, inhibit PDGF-induced collagen production by confluent cultures of porcine myofibroblasts.

9. These *in vitro* alterations of collagen production by PDGF and PTX are not mediated by changes in the gene expression of collagen $\alpha 1$(I).

10. The inhibitory effects of PTX on PDGF-induced collagen production are not mediated by cAMP.

11. PDGF-induced collagen production and proliferation are controlled by different intracellular signal transduction mechanisms.

Future Work

Although several important questions on the mechanisms of collagen accumulation in liver fibrosis have been answered in this thesis, this process is still poorly understood. Several attempts to determine the levels of collagenase and TIMP-2 gene expression in the pig model of fibrosis were not succesful, possibly due to a low homology between the human cDNA probes and the porcine mRNA. Measurement of actual collagenase activity in liver biopsies from these animals may provide an alternative means by which to assess the overall status of the collagenase enzymes in the yellow phosphorus treated pigs. This would also allow us to determine if the effects of PTX extend to improving the collagenase activity in these animals.

Pre-incubation of MCM from fibrotic pigs with anti-PDGF antibody reduced the collagen-stimulating ability of the MCM by approximately 50%. Although TGFβ is generally considered to be the major inflammatory cytokine to induce collagen production in liver fibrosis (Gressner and Bachem, 1995; Friedman, 1993), it is unknown how much activity TGFβ contributes to the fibrotic pig MCM-induced collagen production *in vitro*, and whether or not PTX alters the effects of TGFβ on collagen production *in vivo* or *in vitro*.

The mechanism by which PTX inhibits PDGF-induced collagen production is still unknown. PTX is known to inhibit NF-κB activity (Biswas *et al*, 1993) and

PDGF is known to promote NF-κB actions (Olashaw *et al*, 1992). Although no NF-κB binding sites have been reported in the promoter region of any collagen genes, it is possible that this transcription factor alters the expression of enzymes that regulate the post-translational modifications of the procollagen peptides. Neither PDGF nor PTX was found to affect directly the expression of the collagen α1(I) gene *in vitro* suggesting that their effects on collagen production are post-translational. Prolyl hydroxylase and protein disulphide isomerase activites are both affected by cytokines (Kawaguchi *et al*, 1992) and provide a likely site at which PDGF and PTX can mediate their actions, possibly via the NF-κB transcription factor.

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IMAGE EVALUATION
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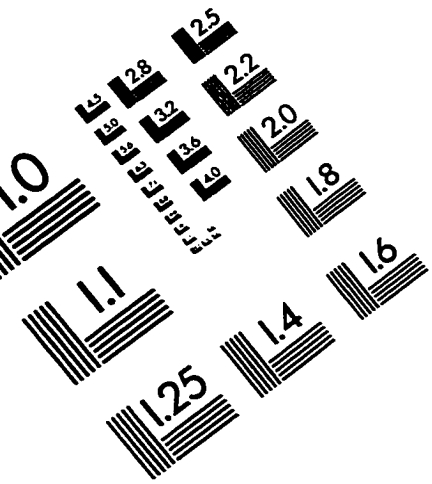

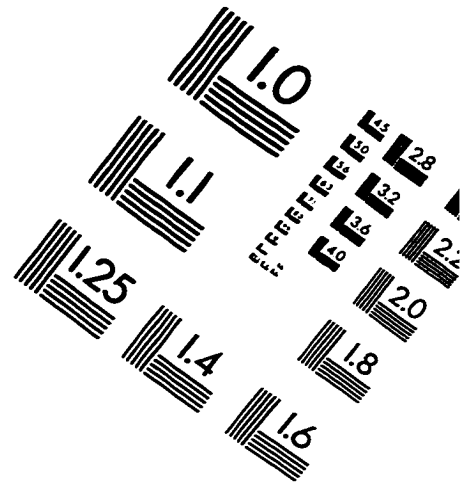

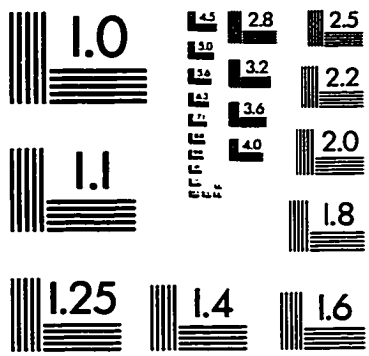

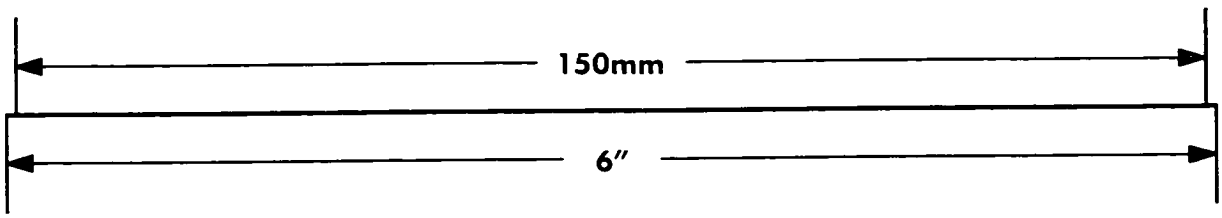

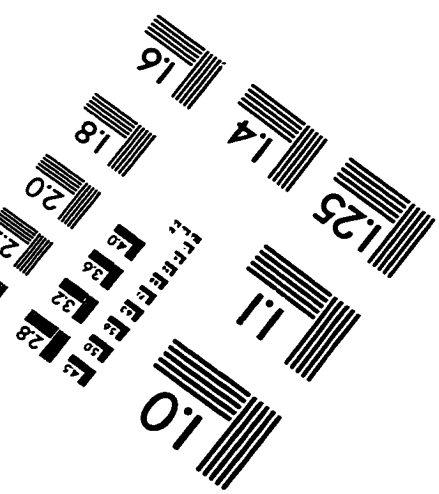

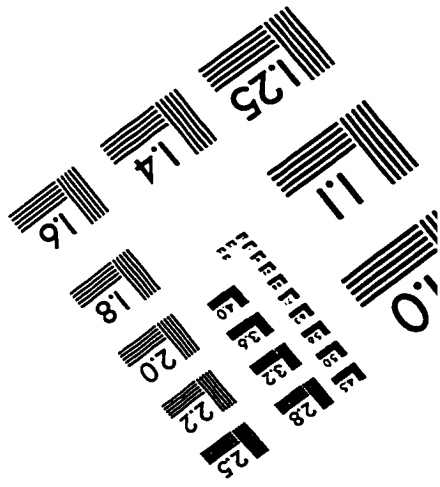

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