Coordinated Induction of Apoptosis and Extracellular Matrix Resorption Reverses Severe Pulmonary Vascular Disease

by

Kyle Northcote Cowan

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

© Copyright by Kyle Northcote Cowan 2000
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
Coordinated Induction of Apoptosis and Extracellular Matrix Resorption
Reverses Severe Pulmonary Vascular Disease

Kyle Northcote Cowan
Doctor of Philosophy, 2000
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

ABSTRACT

Progressive pulmonary vascular disease (PVD) is characterized by smooth muscle cell (SMC) proliferation and migration leading to the formation of occlusive lesions. Reported in this thesis is the co-distribution of the extracellular matrix (ECM) glycoproteins fibronectin with migrating SMC and tenascin-C with proliferating SMCs in pulmonary arteries (PAs) from patients with congenital heart defects and pulmonary hypertension.

As tenascin-C is regulated by mechanical stress, we investigated the response of SMCs in the whole vessel using either normal or hypertrophied PAs from piglets and rats embedded in ‘attached’ versus ‘floating’ collagen gels. While no difference in medial wall thickness was observed with normal PAs, hypertrophied rat PAs showed increased elastase, matrix metalloproteinase (MMP)-2, tenascin-C, and SMC proliferation correlating with progressive medial thickening. In contrast, reduction of these features on floating collagen was related to induction of SMC apoptosis and loss of excess ECM, causing regression of the thickened media.

This response was recapitulated by direct inhibition of either serine elastases or MMPs. However, selective repression of tenascin-C by antisense induced SMC apoptosis and
arrested vascular thickening, but failed to induce regression. We related this to the concomitant expansion of a tenascin-independent SMC population which received pro-proliferative signals similar to tenascin-C from an alternative α3β3 ligand, osteopontin. Induction of apoptosis and regression by proteinase inhibition was related to the unclustering of β3 integrins following loss of α3β3 ligation to both tenascin-C and osteopontin.

The regression of PA medial thickening documented in organ culture was then extended to the intact rat where the reversal of fatal PVD by oral serine elastase inhibitors resulted in normalization of PA pressure and morphometric features.

Taken together, these data demonstrate a critical role for β3 integrin ligating ECM survival factors, like tenascin-C, in the progression of PVD. Regulating the availability of such factors, through the control of proteinase activity or accessibility of α3β3 receptors, may offer novel therapeutic options for this disease.
ACKNOWLEDGEMENTS

I extend heartfelt thanks to my supervisor, Dr. Marlene Rabinovitch, for her enthusiastic encouragement and direction throughout the course of my research, and for sharing her research vision. I have learned by her example that, by looking beyond traditional boundaries, approaching a problem from all perspectives and, through conviction, a solution to any dilemma can be found. Dr. Rabinovitch has been an inspirational mentor to myself and all that train under her supervision.

Profound thanks are owing to Dr. Peter Lloyd Jones who, while being a good friend, was instrumental in nurturing my appreciation and approach to research in the ‘early years’, when I was a summer student under his supervision, and for his philosophical, technical, and critical contributions to the work presented in this thesis. Students and collaborators have also been vital in the progression of this work; these individuals include Adrian Heilbut, Dr. Tilman Humpl, Catherine Lam, Christopher Mar, Jeremy Gilbert, Dr. Y. Kaneda, Dr. D. Osmond, and Dr. S. Ito. I would also like to acknowledge the significant contribution of Dr. S. Hassan E. Zaide for the many research discussions, meeting excursions and, most importantly, his friendship.

Thanks go to all members of the Division of Cardiovascular Research for helping to create an environment in which ideas and new approaches can flourish. For assistance with manuscripts, meetings, scholarships, or anything at all, I would like to thank Joan Jowlabar, Judy Matthews, and Judy Edwards. I am also grateful to Lily Morikawa from the Department of Pathology, for her reliable excellence in cutting and Movat pentachrome staining tissue sections, as well as the members from Laboratory Animal Services at the Hospital For Sick Children.

I would like to acknowledge the supervision of my advisory committee members who have willingly given of their time throughout the M.Sc., M.Sc.-to-Ph.D. transfer, and Ph.D. portions of my training. These individuals include Dr. Lowell Langille, Dr. Jay Cross, Dr. Avrum Gotlieb, and Dr. Mel Silverman. In addition, thanks go to Dr. Shaf Keshavjee for being my M.Sc.-Ph.D. transfer examiner and for joining my advisory committee during my Ph.D. work. I’ve also greatly appreciated the involvement of Dr. Brad Strauss, as my Ph.D. internal examiner, and Dr. Kurt Stenmark, who made the trip from Denver to be my external examiner for my Senate Defense.

I am also endlessly grateful to my friends for providing sanity breaks in tough times, patiently learning more about my research than anyone should endure, and being instrumental in the celebration of successes.

My deepest thanks go to my family, my mother Joan, my brother and sister-in-law, Bryce and Paisley, and my father and step-mother, Jim and Alice. Your contributions exceed measure and my ability to appropriately acknowledge them here. This degree of appreciation is also extended to my late grandparents, Barbara and John Northcote, who were immeasurably instrumental in my life and to whom my successes are dedicated.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xv</td>
</tr>
<tr>
<td><strong>GENERAL INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>I. Clinical Presentation of Pulmonary Hypertension</td>
<td>2</td>
</tr>
<tr>
<td>Pathology (Structural Features)</td>
<td>5</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>7</td>
</tr>
<tr>
<td>Current Therapy</td>
<td>11</td>
</tr>
<tr>
<td>A Closer Look at the Hypertensive Vessel</td>
<td>13</td>
</tr>
<tr>
<td>II. Models of Pulmonary Vascular Disease</td>
<td>14</td>
</tr>
<tr>
<td>Overview</td>
<td>14</td>
</tr>
<tr>
<td>The Monocrotaline Model of Pulmonary Vascular Disease</td>
<td>21</td>
</tr>
<tr>
<td>III. Role of Serine Elastases and Matrix Metalloproteinases</td>
<td>25</td>
</tr>
<tr>
<td>in Vascular Pathobiology</td>
<td></td>
</tr>
<tr>
<td>Serine Elastases</td>
<td>25</td>
</tr>
<tr>
<td>Matrix Metalloproteinases</td>
<td>30</td>
</tr>
</tbody>
</table>
IV. Tenascin-C and Osteopontin as Vascular Cell Survival Factors

Tenascin-C as a Vascular Cell Survival Factor
Could Alternative β3 Integrin Ligands Replace Tenascin-C?

V. Apoptosis in Vascular Pathology

Apoptosis as a Potential Therapeutic Strategy

HYPOTHESIS

OBJECTIVES

CHAPTER ONE

Tenascin-C, Proliferation and Subendothelial Fibronectin in Progressive Pulmonary Vascular Disease

Acknowledgements
Introduction
Methods
Results
Discussion

CHAPTER TWO

Regression of Hypertrophied Rat Pulmonary Arteries in Organ Culture is Associated with Suppression of Proteolytic Activity,
CHAPTER FIVE

Complete Reversal of Fatal Pulmonary Hypertension in Rats by a Serine Elastase Inhibitor

Acknowledgements
Introduction
Methods
Results and Discussion

SUMMARY AND FUTURE DIRECTIONS

REFERENCES
LIST OF TABLES

Table 1.1  Clinical, Hemodynamic, and Lung Biopsy Findings. 78

Table 1.2  Expression and Localization of Tenascin-C, Proliferating Cell Nuclear Antigen, Epidermal Growth Factor, and Cellular Fibronectin. 82
LIST OF FIGURES

0.1 Heath-Edwards classification of pulmonary vascular changes. 9

0.2 Schematic summary of the putative mechanism involved in the initiation and progression of pulmonary vascular disease. 32

0.3 Schematic of potential pathways by which serine elastases may regulate matrix metalloproteinase (MMP)-2. 36

0.4 Hypothetic model for the regulation and function of tenasin-C in vascular smooth muscle cells. 39

0.5 Schematic of the modular extracellular matrix glycoprotein tenasin-C. 42

0.6 Schematic representation of pulmonary artery smooth muscle cells on attached and floating collagen gels, and the effects on tenasin-C production. 45

0.7 Tenascin-C acts as a vascular smooth muscle cell survival factor. 49

0.8 Hypothetical schematic illustrating the reversal of pulmonary vascular disease by the induction of smooth muscle cell apoptosis in response to suppression of tenasin-C either directly or indirect, by serine elastase or matrix metalloproteinase inhibition. 59

1.1 Representative photomicrographs of lung biopsy tissue following Movat pentachrome staining. 76
1.2 Representative photomicrographs showing immunoperoxidase staining for tenascin-C, proliferating cell nuclear antigen and epidermal growth factor in graded lung biopsy tissue sections.

1.3 Representative photomicrographs showing immunoperoxidase staining for cellular fibronectin (FN) in graded lung biopsy tissue sections.

1.4 Representative photomicrographs showing immunoperoxidase staining for α-smooth muscle actin in graded lung biopsy tissue sections.

1.5 Representative photomicrographs showing immunoperoxidase staining for T-cells and macrophages in a typical Grade IVC lesion showing obstructive neointimal formation.

1.6 Representative photomicrographs showing Apoptag and DAPI staining in a vessel showing a Grade IB lesion, and in full thickness human skin.

2.1 Matrix metalloproteinase activity in porcine pulmonary artery organ culture tissue collected from attached and floating cultures.

2.2 Tenascin-C deposition by western immunoblot in porcine pulmonary artery organ culture tissue collected from attached and floating cultures.

2.3 Photomicrographs of porcine pulmonary artery organ culture tissue sections, from attached and floating cultures and immunostained for tenascin-C, PCNA, and following TUNEL assay.
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>Changes in medial wall thickness and elastic lamina of rat pulmonary arteries in attached and floating organ culture.</td>
<td>119</td>
</tr>
<tr>
<td>2.5</td>
<td>Elastase and matrix metalloproteinase activity in rat pulmonary arteries after attached versus floating organ culture.</td>
<td>121</td>
</tr>
<tr>
<td>2.6</td>
<td>Tenascin-C mRNA, protein and immunolocalization in rat pulmonary arteries from attached and floating cultures.</td>
<td>123</td>
</tr>
<tr>
<td>2.7</td>
<td>Proliferating cell nuclear antigen positive cells in rat pulmonary artery organ culture tissue from attached versus floating cultures.</td>
<td>125</td>
</tr>
<tr>
<td>2.8</td>
<td>Apoptosis in floating versus attached rat pulmonary arteries in organ culture.</td>
<td>127</td>
</tr>
<tr>
<td>3.1</td>
<td>Tenascin-C Antisense cRNA.</td>
<td>153</td>
</tr>
<tr>
<td>3.2</td>
<td>Elastase and Matrix Metalloproteinase Activity.</td>
<td>155</td>
</tr>
<tr>
<td>3.3</td>
<td>Regulation of Tenascin-C by Proteinase Inhibitors.</td>
<td>157</td>
</tr>
<tr>
<td>3.4</td>
<td>Apoptosis in Hypertensive and Normotensive Pulmonary Arteries.</td>
<td>159</td>
</tr>
<tr>
<td>3.5</td>
<td>Quantitative Analysis of Apoptosis and Proliferation.</td>
<td>161</td>
</tr>
<tr>
<td>3.6</td>
<td>Proteinase Inhibitors and Regression of Vascular Hypertrophy.</td>
<td>163</td>
</tr>
</tbody>
</table>
3.7 Distribution of Tenascin-C Antisense Transfection and Suppression of
Tenascin-C. 165

3.8 Tenascin-C Antisense Transfection Induces Apoptosis. 167

3.9 Effect of Tenascin-C Antisense on Proliferation and Wall Thickness. 169

3.10 A Tenascin-Independent Smooth Muscle Cell Population. 171

3.11 Tenascin-C Antisense Induces Upregulation of Osteopontin with Proliferating Smooth Muscle Cells. 173

3.12 Suppression of Osteopontin by Proteinase Inhibition. 175

3.13 Osteopontin and αvβ3 Mediated Smooth Muscle Cell Survival. 177

4.1 Induction of A10 smooth muscle cell apoptosis by floating and matrix Metalloproteinase inhibition. 195

4.2 Apoptotic cell counts correlate with propidium iodide flow cytometry and cell number. 197

4.3 The effect of β3 integrin blockade on A10 smooth muscle cell survival. 199

4.4 Changes in the localization of β3 integrins with apoptosis induction. 201

4.5 β3 integrin clustering rescues A10 smooth muscle cells from apoptosis. 203
5.1 Inhibition of monocrotaline-induced elastolytic activity results in survival, and is related to a reversal of pulmonary hypertension and arrest in progression of right ventricular hypertrophy.

5.2 Elastase inhibition reverses pulmonary artery muscular changes and prevents further loss of small vessels.

5.3 Elastase inhibition arrests tenascin-C accumulation and proliferation, and induces apoptosis and loss of extracellular matrix.

6.1 Schematic representations of the consequence of β3 integrin clustering versus unclustering to apoptosis.
LIST OF ABBREVIATIONS

A10 cells: rat aortic smooth muscle cell line
α1-PI: alpha1-proteinase inhibitor
ADAM: a disintegrin and metalloproteinase
AML: acute myelogenous leukemia
ANOVA: analysis of variance
APMA: (4-aminophenyl) mercuric acetate
ASD: atrial septal defect
ATP: adenosine triphosphate
Att: attached
AVSD: atrioventricular septal defect
bFGF: basic fibroblast growth factor
bp: base pair
BrdU: bromo-deoxyuridine
BSA: bovine serum albumin
CAT: chloramphenicol acetyltransferase
cDNA: complementary deoxyribonucleic acid
CHD: congenital heart disease
CHO: chinese hamster ovary
CO₂: carbon dioxide
cRNA: complementary ribonucleic acid
DAB: 3′3-diaminobenzidine
DAPI: 4′6-diamidino-2-phenylindole
DMSO: dimethylsulphoxide
DNA: deoxyribonucleic acid
ECM: extracellular matrix
EDRF: endothelial derived relaxing factor
EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
EGFr: epidermal growth factor receptor
eNOS: endothelial nitric oxide synthase
ERK: extracellular-signal related kinase
EVE: endogenous vascular elastase
EVG: elastin-van Giesen
FAC: focal adhesion complex
FACS: fluorescent activated cell sorter
FAK: focal adhesion kinase
FBS: fetal bovine serum
FGF: fibroblast growth factor
Fl: floating
FN: fibronectin
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
HBSS: Hank’s buffered saline solution
HCl: hydrochloric acid
HE: haematoxylin eosin
HIF: hypoxia inducible factor
HMG: high mobility group
HVJ: haemagglutinating virus of Japan
IAP: inhibitor of apoptosis protein
Ig: immunoglobulin
IGF: insulin-like growth factor
IgG: immunoglobulin G
IgM: immunoglobulin M
kb: kilobases
kD: kilodalton
kDa: kilodalton
LDH: lactate dehydrogenase
LDL: low density lipoprotein
LSD: least significant difference
LV: left ventricle
LV+S: left ventricle plus septum
MAPK: mitogen activated protein kinase
MCAF: monocyte chemotactic and activating chemokine
MCP: monocyte chemoattractant protein
MCT: monocrotaline
MFO: mixed function oxidase
MMP: matrix metalloproteinase
MT-MMP: membrane type matrix metalloproteinase
mRNA: messanger ribonucleic acid
MW: molecular weight
NACI: sodium chloride
NF-κB: nuclear factor kappa B
NO: nitric oxide
NOS: nitric oxide synthase
OPN: osteopontin
PA: pulmonary artery
PBS: phosphate buffered saline
PBSA: phosphate buffered saline with 1% bovine serum albumin
PCNA: proliferating cell nuclear antigen
PDGF: platelet derived growth factor

xvii
PEG: polyethylene glycol
PH: pulmonary hypertension
PKB: protein kinase B
PKG: protein kinase G
Ppa: mean pulmonary artery pressure
PVD: pulmonary vascular disease
Qp:Qs: pulmonary to systemic flow ratio
RGD: arginine-glycine-aspartate
rRNA: ribosomal ribonucleic acid
RV: right ventricle
RVH: right ventricular hypertrophy
SEM: standard error about the mean
SHR: spontaneously hypertensive rats
SMC: smooth muscle cell
S/P: statis post
TdT: terminal deoxynucleotidyltransferase
TGA: transposition of the great arteries
TGF-β: transforming growth factor beta
TIMP: tissue inhibitor of metalloproteinase
TN: tenascin-C
TNFα: tumour necrosis factor alpha
TUNEL: terminal deoxuridine nick end labeling
Tyr-P: tyrosine-phosphorylated
USP: upstream stimulatory factor
UTP: uridine triphosphate
VSD: ventricular septal defect
vWF: vonWillebrand factor

xviii
GENERAL INTRODUCTION

In this thesis, we relate increasing deposition of the matrix glycoprotein tenascin-C (TN) to the smooth muscle cell (SMC) proliferation which occurs in the oblitative progressive form of pulmonary vascular disease (PVD) observed in patients with congenital heart defects (CHD) characterized by elevated main pulmonary artery (PA) pressure and flow. To further investigate the mechanistic relationship, studies were carried out first with normal main porcine pulmonary arteries (PAs) in organ culture and then with hypertensive rat PAs where we showed that TN deposition was related to increased activity of proteolytic enzymes, elastases and matrix metalloproteinases (MMPs), and that stress unloading could reverse medial thickness by suppression of these enzymes, resulting in a decrease in TN and induction of apoptosis. We then showed that the effects of stress unloading could be reproduced by culturing PA tissues with proteinase inhibitors. Suppression of TN with an antisense strategy was also effective in inducing the apoptotic response. However, a subpopulation of SMCs continued to proliferate accounting for a failure to reverse medial hypertrophy when TN was selectively targeted. The ability of this SMC subpopulation to survive in the absence of TN was related to their increased production of an alternative extracellular matrix (ECM) β3 integrin ligand, osteopontin (OPN). Indeed, blockade of β3 integrins in PA organ cultures recapitulated the regression of hypertrophy by inducing SMC apoptosis and resorption of excess ECM proteins. Cell culture studies revealed that survival, through β3 integrin ligation, was related to clustering of this integrin subtype. Having demonstrated the ability to regress pulmonary vascular lesions with either proteinase inhibitors or through β3 integrin blockade, we determined whether these strategies might be effective in the intact animal. Using orally bioavailable serine elastase inhibitors, survival was induced in rats with a fatal form of pulmonary vascular disease. The underlying mechanism was associated with suppression of TN and induction of SMC apoptosis
causing reversal of increased muscularity of peripheral arteries and normalization of main PA pressure.

Therefore the Introduction to this thesis will be divided into five sections. In the first section, the clinical presentation of pulmonary hypertension: its morphological features, diagnosis, and current therapy will be reviewed. The second section will survey a variety of experimental models of pulmonary hypertension, with an emphasis on literature pertaining to the use of monocrotaline in rats and our own studies suggesting a pivotal role for an elastase in the initiation and progression of pulmonary vascular disease. The third section describes evidence for the role of proteolytic enzymes in vascular pathology, focusing on serine elastases, specifically the vascular elastase described by our group, and matrix metalloproteinases (MMPs). The fourth section addresses vascular ECM survival factors, focusing on work by our group characterizing a role for TN within the vasculature and describing its regulation by proteolytic activity, as well as the potential ability of OPN, an alternative β3 ligand, to replace TN, as part of the SMC response to TN withdrawal. Finally, apoptosis, pertaining to vascular pathology, is introduced in the fifth section, which also includes a discussion of the potential to harness this process in therapeutic strategies aimed at preventing vascular disease.

I. Clinical Presentation of Pulmonary Hypertension

Pulmonary vascular disease and associated pulmonary hypertension (PH) was probably first described in 1897 by Victor Eisenmenger who reported a 32 year-old man that presented with unexplained exercise intolerance and cyanosis, and died of congestive heart failure after an episode of hemoptysis (Eisenmenger, 1897). Pulmonary hypertension, characterized by the progressive structural remodeling of the pulmonary vascular bed leading to elevated pulmonary arterial pressure and pulmonary vascular resistance, has since
been documented to be induced by a variety of cardiac and non-cardiac conditions [reviewed in (Rabinovitch, 1998)].

As a disease affecting both the heart and lungs, the pathogenesis of pulmonary hypertension can be initiated by increases in pulmonary blood flow and pressure developing secondary to a congenital heart defect (CHD) with a left to right shunt (Eisenmenger, 1897; Civin and Edwards, 1950; Rudolph and Nadas, 1962; Rabinovitch, 1998). One of the best examples is transposition of the great arteries (Viles, et al., 1969), especially when associated with a large ventricular septal defect (Newfeld, et al., 1974) or a patent ductus arteriosus (Waldman, et al., 1977). Infants with a large ventricular septal defect alone also develop PVD and often have early signs of the disease that are detectable within the first 6 months of life (Iverson, et al., 1966). The development of PH has not been restricted to CHD causing elevated pulmonary blood flow. In patients with congenital mitral stenosis (Collins, et al., 1977), total anomalous pulmonary venous connection (Ferencz and Dammann, 1957; Haworth and Reid, 1977b; Newfeld, et al., 1980), hypoplastic left heart syndrome (Naeye, 1962; Haworth and Reid, 1977a). or rheumatic mitral stenosis (Wagenvoort and Wagenvoort, 1982), PH has resulted from elevated pulmonary venous pressure, although the frequency and severity with which it occurs is considerably less than with high flow lesions (Rabinovitch, 1995).

In addition to CHD, PH can be induced by hypoxia. While the effects of acute hypoxia are often mild and temporary (Hultgren, et al., 1965), the variability in individual response can sometimes be manifest as severe PH with high-altitude pulmonary edema (Scoggin, et al., 1977), which occurs more commonly in children (Rabinovitch, 1998). This severe response may be attributable to the development of edema, formation of microemboli, inadequate diuresis, the abnormal endothelial metabolism of factor VIII, or it may have an immune basis (Rabinovitch, 1995). Prolonged hypoxic exposure, as observed in individuals living at
extreme altitudes, leads to a chronic elevation in PA pressure that is not correctable by oxygen administration (Arias-Stella and Saldana, 1963; Vogel, et al., 1964; Grover, et al., 1966). rather the pulmonary hypertension is related to an abnormal muscularization of peripheral arteries when compared structurally to PAs from individuals at lower altitudes. Pulmonary hypertension can also occur secondary to parenchymal lung diseases including cystic fibrosis, bronchopulmonary dysplasia (Hislop and Haworth, 1990), and infrequently in children with asthma (Griffin, et al., 1959). It is a common complication of restrictive lung disease, such as diffuse interstitial fibrosis (Hamman-Rich) (Baar and Braid, 1957), infiltrative lung tumors (Altemus and Lee, 1967), and collagen vascular disease (Rosenberg, et al., 1979). Neuromuscular disorders affecting the chest wall, such as Duchenne muscular dystrophy, poliomyelitis, and Werdnig-Hoffman disease, and diseases affecting the vertebrae and rib cage, such as scoliosis, may so impair ventilation causing PH [reviewed in (Rabinovitch, 1998)].

In the newborn, pulmonary hypertension is associated with the following: hypoplasia of the lung and underdevelopment of the vascular bed, as a feature of congenital diaphragmatic hernia, scimitar syndrome, absence of the phrenic nerve, and asphyxiating thoracic dystrophy (Rabinovitch, 1998). It can also occur when there is maladaptation to postnatal physiology, due to the presence of perinatal stresses including hemorrhage, hypoglycemia, aspiration, or hypoxia, and maldevelopment of the pulmonary vascular bed in utero from an unknown etiology (Rabinovitch, 1995).

In addition, thromboembolic diseases, such as deep vein thrombosis (Nguyen, et al., 1986), right-sided endocarditis and right atrial myxoma (Heath and Mackinnon, 1964) may be sources of pulmonary emboli which can organize and lead to obstruction and pulmonary hypertension. Severe liver disease producing cirrhosis and intrahepatic portal hypertension, and portal vein thrombosis, producing extrahepatic portal hypertension, have also been
associated with the development of PH (Levine, et al., 1973; Edwards, et al., 1987; Silver, et al., 1992) by a mechanism that is unclear. The ingestion of various 'toxins' is also known to be associated with PH (Rabinovitch, 1998). Such substances include anorexigens, like aminorex, which resembles epinephrine in its chemical structure and which suppresses the appetite by acting centrally on the brain (Peters and Gourzis, 1974). These agents, which include the fenfluoramine compounds, most recently, dexfenfluramine, which is a serotonin antagonist (Abenhaïm, et al., 1996) are known to cause symptoms of right-sided heart failure in 10% of patients within 6 to 12 months of initial administration (Milzoch, 1980). Ingestion of pyrrolizidine alkaloids such as bush tea causes hepatic veno-occlusive disease and a similar compound, monocrotaline (MCT), causes pulmonary vascular disease in rats (Meyrick, et al., 1980). Having ruled out all known causes of PH, a patient is then diagnosed with 'idiopathic' or unexplained PH. This form of PH, which may or may not be observed in families, is more common in females (Bourdillon and Oakley, 1976; Fujii, et al., 1981). Severe structural abnormalities develop in the pulmonary vessels that rapidly progress, becoming invariably fatal in the absence of treatment (Rogge, et al., 1966; Hendrix, 1974; Loyd, et al., 1995; Rabinovitch, 1998).

Pathology (Structural Features)

Regardless of the etiology of PH, the structural abnormalities observed during the progression of this disease are similar. Rabinovitch and colleagues quantitatively assessed the morphology of pulmonary arteries in lung biopsy tissue obtained from infants with CHDs and concluded that the severity of altered growth and development of the pulmonary vascular bed correlated with the hemodynamic state of the pulmonary circulation (Rabinovitch, et al., 1978). Evolving as a series of stages, the earliest alteration is precocious muscularization of normally non-muscular peripheral arteries including those at alveolar wall level that are normally non-muscular even in the adult. This process is initiated by an increase in pulmonary blood flow and is related to differentiation of SMCs from
precursor cells, pericytes and intermediate cells, as described by Meyrick and Reid on the basis of ultrastructural analysis of lung biopsy tissue from children with CHDs (Meyrick and Reid, 1978; Meyrick and Reid, 1980b). The stimulus for precursor to SMC differentiation may be 'stretch' associated with chronic high flow and high pressure, given that arteries become more muscular as they increase in size, i.e. external diameter (Rabinovitch, 1995). This feature, together with a mild increase (less than 1.5 times normal) in the wall thickness of normally muscular arteries, is considered grade ‘A’, as the first of three grades established by Rabinovitch et al (Rabinovitch, et al., 1978).

A greater extension of muscle along normally non-muscular PAs, when accompanied by a more severe medial hypertrophy of normally muscular PAs (>1.5 times normal), denotes grade B. If the latter is still less than 2 times normal, a 'mild grade B' is assigned, while a greater degree of hypertrophy (≥2 times normal wall thickness) warrants the subgrade, severe grade B (Rabinovitch, 1998). This pronounced increase in medial thickness is attributed to the hypertrophy and hyperplasia of resident SMCs, the deposition of ECM components and intercellular connective tissue, and is associated with an increase in mean pulmonary artery pressure. The PA pressure is generally greater than half that of the systemic level with severe grade B.

The highest morphometric grade, grade C, has, in addition to the findings of "severe grade B", a reduction in arterial concentration and sometimes in artery size as well. Patients with this feature exhibit an increase in pulmonary vascular resistance. When the artery number becomes less than half of normal, severe grade C is assigned. The reduced arterial concentration is presumably due to the failure of normal vascularization, although resorption and loss of existing vessels distal to occluded arteries with severe neointimal formation may also occur (Rabinovitch, 1998).
Morphometric grades A and B are refinements of grade I from an older, pre-existing grading system proposed by Heath and Edwards in 1958 (Heath and Edwards, 1958). This grading system first suggested a progression of structural changes (Fig. 0.1), where grade I represented medial hypertrophy. Grade C on the morphometric system is a new feature, but one that commonly accompanies grades II and III of the Heath-Edwards classification, namely cellular and occlusive intimal proliferation, respectively. Grade IV was identified by early to advanced arterial dilation, while grades V and VI are angiomatoid formation and fibrinoid necrosis, respectively. While these grading systems are designed to describe pulmonary vascular changes, the general pathological features, specifically medial hypertrophy and neointimal formation are seen with many systemic vascular diseases including atherosclerosis and post-cardiac transplant coronary arteriopathy (Ross, 1986; Ip, et al., 1990; Billingham, 1992).

**Diagnosis**

When pulmonary hypertension is of unexplained etiology, patients experience on average, 2 to 5 symptomatic years prior to diagnosis, as reported by Rich and colleagues (Rich, et al., 1987). Symptoms like exercise-intolerance are first apparent due to lack of vascular reserve, and can include dypsea, orthopnea and chest pain similar to angina (Nihill, 1995). As PH is accompanied by right ventricular hypertrophy (RVH) and dysfunction, early satiety, lower extremity edema and fluid overload are often present. Limitations in the cardiac response to increased demand may result in presyncope or syncope.

Early investigation into a diagnosis of PH involves non-invasive approaches like electrocardiography which, in a patient with PH, often reveals right-sided pressure overload, i.e., P-pulmonale, right-axis deviation, right ventricular hypertrophy, and a right
Figure 0.1

*Heath-Edwards classification of pulmonary vascular changes.*  
**A:** Grade I: medial hypertrophy. EVG, elastin-van Giesen stain, X150.  
**B:** Grade II: cellular intimal proliferation in an abnormally muscular artery. EVG, X250.  
**C:** Grade III: occlusive changes. Media is thickened due to fasciculi of longitudinal muscle, and vessel is all but occluded by fibroelastic tissue. EVG, X150.  
**D:** Grade IV: dilation. Vessel is dilated and muscular media is abnormally thin. Lumen is occluded by fibrous tissue. EVG, X150.  
**E:** Grade V: plexiform lesion. There is cellular intimal proliferation; clustered around are numerous thin-walled vessels that terminate as capillaries in the alveolar wall. EVG, X95.  
**F:** Grade VI: acute necrotizing arteritis. A severe reactive inflammatory exudate is seen through all layers of the vessel. HE, haematoxylin eosin stain, X250.  
ventricular strain pattern (Restrepo and Tapson, 1998). Chest radiography can provide an assessment of cardiac size, specifically the degree of RVH, and sometimes identifies precipitating conditions, such as lung abnormalities like pulmonary fibrosis (Kanemoto, et al., 1979). Similarly, pulmonary function testing, like spirometry, has also been found useful for the identification of pulmonary obstructive abnormalities (D'Alonzo and Dantzker, 1997). Two- and three-dimensional echocardiography have been used to estimate RVH from the ratio of measurements of the right ventricular free wall to the left ventricle plus entire septum (Fulton, et al., 1952; Bates, et al., 1996) and degree of 'left-ventricularization' of the right ventricle (Rahlf, 1978), while pulse-wave Doppler can flag PH and can often quite accurately predict PA systolic pressures from the pulmonary regurgitation jet (Jaffe and Weltin, 1992). While estimates of PA pressure can also be acquired using procedures like high resolution computerized tomography (Bergin, et al., 1996), magnetic resonance imaging (Murray, et al., 1994; Helbing, et al., 1995), and fractal analysis (Boxt, et al., 1994), and evidence of thromboembolic disease attained with ventriculography (Jaffe and Ellis, 1974), ventilation-perfusion scanning (D'Alonzo, et al., 1984), and pulmonary arteriography (Shure, et al., 1985; Auger, et al., 1992), often with significant risk, the gold standard for the diagnosis of PH remains the hemodynamic and wedge angiographic assessments (Restrepo and Tapson, 1998).

Wedge angiography assesses the structural state of the pulmonary vascular bed, utilizing a thermodilution balloon catheter (Swan, et al., 1970). The qualitative assessment of the sparsity of arborization of the pulmonary tree, abrupt termination, tortuosity and narrowing of small arteries, and reduced background capillary filling, will indicate at least Heath-Edwards grade III (Nihill and McNamara, 1978). The balloon occlusion pulmonary wedge angiogram technique allows for precise quantitative measurements of the rate of arterial tapering and can be used to predict both the morphometric grades as well as the Heath-Edwards classification (Rabinovitch, et al., 1981). These techniques are not without
limitations and when haemodynamic or structural measurements are difficult to obtain or interpret, quantitative assessments on frozen-section lung biopsy tissue have been successfully applied to predict the potential for reversibility of pulmonary vascular disease (Rabinovitch, et al., 1981).

**Current Therapy**

When PH has developed as a consequence of a primary condition, such as a CHD, surgical or medical treatment of the primary condition is usually associated with regression of the hypertension. The degree to which this occurs with CHD depends upon the age of the patient and the severity of the vascular abnormalities. A study by Rabinovitch et al (Rabinovitch, et al., 1984) correlated pulmonary vascular changes in lung biopsies from children, obtained at the time of CHD correction, with postoperative pulmonary haemodynamic findings. Infants receiving corrective surgery prior to eight months of age regardless of the severity of vascular changes, or older children with relatively mild changes such as grade B medial hypertrophy exhibited reversibility of elevated PA pressures. In the remainder of the patients more severe structural abnormalities were associated with persistent elevation in pulmonary vascular resistance and progressive symptomatology.

In patients with reduced oxygen tension, clinical trials with supplemental oxygen have shown a reduction in mortality [multicenter clinical trial reports (1980; 1981)]. While these trials targeted a reduction in hypoxic pulmonary vasoconstriction, as a way of decreasing chronically elevated PA pressure and resistance, the best result achieved was a stabilization of PA pressure. Similarly, various vasodilator agents have been tried in an effort to reduce the level of PA pressure, relieve symptoms, and retard the disease progression (Rubin, 1997). Calcium channel blockers, like nifedipine and diltiazem, while improving pulmonary vascular resistance, may or may not increase survival (Morley, et al., 1987; Saadjian, et al., 1987; Agostoni, et al., 1989).
On the basis of encouraging early studies (Rubin, *et al.*, 1990; Barst, *et al.*, 1994), continuous intravenous infusion of prostacyclin has been used, even in the absence of an acute haemodynamic effect, to stabilize patients (Restrepo and Tapson, 1998). This therapy, while not a cure, has improved quality of life and obviated the need for lung transplant in a subgroup of patients, especially children. NO (nitric oxide) therapy has been advocated as a way to achieve both a vasodilatory response by replacing the loss in NO synthase activity in PH patients (Giaid and Saleh, 1995). The delivery of NO remains an obstacle and recent clinical trials indicate no improvement in mortality (Neonatal Inhaled NO Study Group, 1997; Roberts, *et al.*, 1997). As patients on NO appear at risk for thromboembolism, NO has been given in conjunction with anticoagulants with limited success (Fuster, *et al.*, 1984; Barst, *et al.*, 1996). When a patient is refractory to these treatments, atrial septostomy has been performed to relieve the right ventricular hypertension and tendency to syncope, right heart failure and sudden death (Austin, *et al.*, 1964; Rich and Lam, 1983).

A Closer Look at the Hypertensive Vessel

In 1986, Rabinovitch et al applied scanning and transmission electron microscopy to lung biopsy specimens from patients with CHDs to analyze the PA endothelium for alterations in surface characteristics and intracytoplasmic composition which might reflect abnormal function. These studies addressed the hypothesis that heightened pulmonary vascular reactivity resulted from endothelial dysfunction and that this dysfunction was related to the progression of PVD (Rabinovitch, et al., 1986). These investigators found that the normal 'corduroy' pattern of the endothelium observed on scanning electron microscopy was altered in hypertensive vessels, becoming tortuous, in that the endothelial cells formed winding ridges and deep gorges. This endothelial surface could slow down or trap marginating blood cells such as leukocytes which release vasoconstrictive substances, like thromboxane A2 and B2, both of which have been described as elevated in PH patients (Christman, et al., 1992; Fuse and Kamiya, 1994). Platelet aggregates could also form, blocking small blood vessels. As well, alterations in mechanotransducible forces leads to abnormal endothelial expression of genes associated with proliferation of SMCs (PDGF) (Resnick and Gimbrone, 1995) and enhanced connective tissue deposition (TGF-β family) (Topper, et al., 1996). Indeed, further studies revealed that the morphologically abnormal endothelial cells were also functionally altered in that they produced elevated amounts of a high-molecular weight form of vonWillibrand factor (vWF) and decreased antithrombin III which promotes platelet aggregation and thrombus formation (Rabinovitch, et al., 1987; Turner-Gomes, et al., 1992).

More recent studies, by Dihn et al (Dihn-Xuan, et al., 1990) and Celermajer et al (Celermajer, et al., 1993), have shown a loss of production of the vasodilator, endothelial-derived relaxing factor (EDRF) or NO, in PH patients, while other groups have reported heightened circulating levels of the vasoconstrictor, endothelin (Yoshibayashi, et al., 1991; Adatia and Haworth, 1993). The earlier electron microscopic study by Rabinovitch
(Rabinovitch, et al., 1986) also described an altered internal structure of the endothelium, noting cytoskeletal reorganization in the form of an increased density of microfilament bundles, and a heightened metabolic capacity, suggested by the abundant rough endoplasmic reticulum. In addition, these ultrastructural studies revealed that the underlying subendothelium was markedly altered, with the internal elastic lamina showing evidence of degradation and areas of neosynthesis. While groups have reported modulation of ECM content related to elevated procollagen synthesis (Botney, et al., 1993), and tropoelastin (Prosser, et al., 1989) and fibronectin (FN) expression (Botney, et al., 1992), the mechanism of elastin degradation would subsequently be investigated using experimental models of PVD, where it would prove to be pivotal to disease progression.

II. Models of Pulmonary Vascular Disease

Numerous animal models have been used to recapitulate the pathophysiology of pulmonary vascular disease. In this section, an overview will be provided, together with a section devoted to the use of the toxin monocrotaline (MCT) to induce PH, as this is central to this thesis. With many common features linking the cellular and molecular pathophysiology of pulmonary vascular disease regardless of etiology, it may be possible to extend observations made in one model to other models and even to clinical conditions.

Overview

Speculating that high flow alone can cause pulmonary vascular disease, investigators have produced animals models with high pulmonary blood flow. Some studies used banding to reduce flow by 80% through the left PA in rats (Rabinovitch, et al., 1983) while others completely prevented flow either by ligation of the PA or pneumonectomy (Davies, et al., 1982). These approaches failed to induce the severe PH and associated structural changes. Instead, a mild elevation in PA pressure was observed in the unbanded or remaining lung in
association with a mild extension of muscle into peripheral normally non-muscular PAs, slight medial hypertrophy of normally muscular arteries and reduced arterial density. Experiments designed to recapitulate a CHD by creating aortopulmonary shunts, anastomosing the descending thoracic aorta to the pulmonary trunk, in dogs (Blank, et al., 1961) and piglets (Rendas, et al., 1979) achieved greater success in mimicking PVD. In these studies, the exposure to systemic pressure resulted in the development of severe PH associated with increased muscularization of arteries of all sizes and a reduction in arterial concentration, together with a progressive increase in PA pressure. This process could be rapidly reversed in young piglets following surgical closure of an anastomosis after 5 weeks of patency (Rendas and Reid, 1983), or show progressive development when large shunts were left uncorrected in a dog model (Blank, et al., 1961).

A variety of experimental approaches have tried to stimulate the left-to-right shunting of CHDs. Such studies include anastomosing a systemic artery to the lobar pulmonary artery (Fasules, et al., 1994; Schnader, et al., 1996), the creation of a ventricular septal defect in neonatal lambs (Boucek, et al., 1985), or an aortopulmonary shunt in a fetal lamb (Black, et al., 1998). The latter model has very recently been shown to produce an increase in endothelin-converting enzyme-1 and endothelin receptor type A (responsible for vasoconstriction) with concomitant downregulation of the type B receptor (responsible for vasodilation) (Black, et al., 1999). This substantiates the observation of heightened endothelin expression in clinical pulmonary hypertension. Nevertheless, increased endothelial nitric oxide synthase (eNOS) activity, which is inconsistent with clinical reports and elevated cyclic guanine monophosphate (cGMP) levels, have also been documented in this aortopulmonary shunt model (Black, et al., 1998).

The experimental development of pulmonary venous hypertension, via pulmonary vein banding (Silove, et al., 1972; LaBourene, et al., 1990) or after placement of aorta to left
atrial shunts (Hopkins, et al., 1980), has allowed investigation into the effect of pulmonary venous obstruction, and accompanying increased pulmonary venous flow, on the development of PVD. By banding the pulmonary veins of piglets for 3 weeks, LaBourene and colleagues (LaBourene, et al., 1990) have shown that the increase in PA pressure observed is associated with evidence of degradation of the internal elastic lamina and migration of SMCs from the media to the subendothelium.

As observed in the clinical setting, hypoxia has been used to experimentally induce a hypertensive response in the pulmonary vascular bed. Acute hypoxic exposure elicits a vasoconstrictive response in both arterial and venous microvessels owing to the production of vasoconstrictors such as endothelin (Li, et al., 1994), in addition to leukotrienes and thromboxane A2 (Raj and Chen, 1987). Hypoxia also reduces synthesis of vasodilator prostaglandins and prostacyclins. This is in keeping with studies showing that cyclooxygenase inhibitors, like indomethacin, exacerbate vasoconstriction to acute hypoxia in dogs which normally exhibit a blunted response (Hales, et al., 1978). Work from Shaul et al (Shaul, et al., 1992) and Sprague (Sprague, et al., 1992) would, however, support impairment of the EDRF pathway as being more important than cyclooxygenase inhibitors in aggravating the vasoconstrictive response to hypoxia. Post and colleagues (Lippton, et al., 1991; Post, et al., 1992) provide evidence supporting an inactivation of Ca^{2+}-dependent K^+ channels as a key event linking hypoxia to pulmonary vasoconstriction by causing membrane depolarization and subsequent Ca^{2+} entry. More recently, Chen (Chen, et al., 1995) has shown that pre-treatment with bosentan, an orally active antagonist of endothelin-A and -B receptors, completely blocked the pulmonary vasoconstrictor response to acute hypoxia, while guanylyl cyclase-linked atrial natriuretic peptide receptor deficient mice have been shown by Zhao (Zhao, et al., 1999) to have increased susceptibility to acute hypoxia. How this acute vasoactive response is mechanistically linked to the structural changes observed with chronic hypoxic exposure is not completely unknown. Studies, like those of
Rabinovitch (Rabinovitch, et al., 1983) documenting the effects of altered hemodynamics on the initiation of structural remodeling in the pulmonary vascular bed, may provide some direction for further research.

In extensive animal studies by Tucker (Tucker, et al., 1975) the amount of vascular smooth muscle within the peripheral PAs was a major determinant of the response of that species to hypoxia. Differences within a species also exist and have been shown to be dependent on both age and sex, with females less responsive (McMurtry, et al., 1973; Rabinovitch, et al., 1981). In the rat, exhibiting a 'moderate' response, Rabinovitch et al (Rabinovitch, et al., 1979) documented that after a period of three days in hypobaric hypoxia, mean PA pressure and resistance had risen significantly and were associated with extension of muscle along peripheral arteries. Consequently, these structural changes maintain an elevated PA pressure for several hours or more following removal of the rats from the hypoxic environment. After two weeks of hypoxia, the structural changes now include an increase in medial wall hypertrophy and a reduction in arterial concentration relative to alveoli. At this timepoint, mean PA pressure had doubled with a corresponding increase in right ventricular hypertrophy.

Ultrastructural studies by Meyrick and Reid (Meyrick and Reid, 1978) showed that the muscularization of arteries during hypoxia was related to the differentiation of pre-existing cells, pericytes, to form new muscle in the non-muscular arteries. Extracellular matrix and muscle cell accumulation was co-distributed and it is this process that Hislop (Hislop and Reid, 1977) suggested encroached upon the lumen of the peripheral vessels and accounted for their loss. These vessels often failed to return with normoxia, even after a recovery period of eight weeks, indicating that they may have been reabsorbed (Hislop and Reid, 1977). However, the exact mechanism of vessel loss which limits the functional vascular reserve in these animals, remains unknown (Rabinovitch, 1998). During the recovery
period, adult rats normalize their PA pressures together with the regression of structural changes (Rabinovitch, et al., 1981). However, this regression is incomplete, particularly in rats exposed during infancy since they maintain a mean pulmonary artery pressure that is 50% above normal (Rabinovitch, et al., 1981).

The residual structural abnormalities have been related by Meyrick and Reid (Meyrick and Reid, 1980a) to a continuing accumulation of excess elastin and collagen fibres. These investigators suggested that this neo-matrix would alter the function of the vessel wall, restricting growth and making it less compliant. Indeed, the use of anti-fibrotic agents which inhibit collagen synthesis, like beta-aminopropionitrile (Kerr, et al., 1984) and cis-4-hydroxy-L-proline (Poiani, et al., 1990), limit chronic hypoxia-induced PH.

Using newborn calves, Stenmark and coworkers (Stenmark, et al., 1987) simulated high altitude conditions and showed the development of severe and irreversible PH, with suprasystemic PA pressures that produced right-to-left shunting likely through the foramen ovale and patent ductus arteriosus. Morphometric examination indicated a markedly thickened adventitia, as well as increased medial thickness, and distal muscularization of PAs with evidence of cellular proliferation. This vascular thickening was accompanied by neosynthesis of tropoelastin, the alpha 1 procollagen chain, and FN within discrete regions of the vessel wall (Prosser, et al., 1989; Durnowicz, et al., 1994). Suggesting a mechanism underlying this process, Vender et al (Vender, et al., 1987) have reported the release of SMC mitogens from hypoxia-stimulated pulmonary endothelial cells. Stenmark and colleagues (Badesch, et al., 1989; Dempsey, et al., 1990) have identified that insulin-like growth factor (IGF) -1 as well as trace amounts of protein kinase C (PKC) activators act synergistically to stimulate PA SMC proliferation and can, together with release of transforming growth factor (TGF) -8 (Liu and Davidson, 1988), induce elastin and collagen synthesis. Thus, experimental studies using SMC growth inhibitors, like heparin, have been
able to limit development of hypoxic pulmonary vascular disease (Benitz, et al., 1986; Thompson, et al., 1994).

Investigators have attempted to link the observed vasoconstrictive response to acute hypoxia with the initiation of chronic hypoxic structural remodeling. Acute hypoxic exposure is aggravated by indomethacin (Hales, et al., 1978) and is associated with a downregulation of SMC and endothelial cell prostacyclin synthesis (Madden, et al., 1986; Rabinovitch, et al., 1989). Rabinovitch et al (Rabinovitch, et al., 1988) chronically infused hypoxic rats with exogenous angiotensin II which paradoxically abolished chronic hypoxia associated vascular remodeling. As its protective effect was reversed by co-treatment with indomethacin, angiotensin II likely acts by stimulating the synthesis of the vasodilator and SMC growth inhibitor (Owen, 1985) prostacyclin. Endothelin receptor blockade can prevent acute hypoxic vasoconstriction (Chen, et al., 1995) and, in keeping with reports of high endothelin levels in PH patients (Allen, et al., 1993), many groups have now shown that this approach will also prevent the development of chronic hypoxic vascular disease (Chen, et al., 1995; Oparil, et al., 1995; Holm, et al., 1996).

Impairment of the EDRF or NO pathway, observed in acute hypoxia (Shaul, et al., 1992; Sprague, et al., 1992) and in the clinical setting (Dihn-Xuan, et al., 1990; Celermajer, et al., 1993), has also been addressed. Studies in the rat model of chronic hypoxia have, however, reported an increase in eNOS owing to an induction of the eNOS gene by hypoxia inducible factor (HIF-1). This may be a protective response in that eNOS transgenic knockout mice exhibit a more malignant form of PH when exposed to chronic hypoxia. Indeed, Kouyoumdjian and coworkers (Kouyoumdjian, et al., 1994) have shown that the continuous inhalation of NO will protect against development of PH in chronically hypoxic rats. While a variety of other successful approaches exist, like targeting vasodilation through the reactivation of inactive Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels (Weir and Archer, 1995;
Cornfield, et al., 1996), our laboratory has shown that inhibition of serine elastases also effectively reduces chronic hypoxia-induced PH (Maruyama, et al., 1991).

Evidence for the involvement of an elastolytic enzyme has also been provided by models of PH induced by chronic air embolization. Perkett and coworkers (Perkett, et al., 1988; Perkett, et al., 1991) have reported that, in response to air embolism, chronically instrumented sheep showed a sustained rise in pulmonary artery pressure and pulmonary vascular resistance. The resultant arterial structural remodeling, leading to excessive muscularization, was associated with heightened pulmonary vasoreactivity and characterized by endothelial cell injury and the presence of elevated thromboxane B2 and TGF-β levels as well as elastin peptides in the lung lymph (Perkett, et al., 1988; Perkett, et al., 1990; Perkett, et al., 1991; Perkett, et al., 1994). Similar to the hypoxia model, treatment of the sheep with a serine elastase inhibitor, secretory leukoproteinase inhibitor (SLPI), attenuated the disease process (Gossage, et al., 1995).

Toxin-induced models of PVD have also been commonly used. Based on the observation that chronic PH occurs in several human diseases in which there is evidence of chronic or repeated bouts of pulmonary inflammation, Meyrick and coworkers (Meyrick and Brigham, 1986) delivered E. coli endotoxin to seven chronically instrumented sheep for 14 weeks. Electron microscopy revealed a persistent inflammation associated with an accumulation of granulocytes, lymphocytes, and monocytes in the lungs of these animals. Morphologically the PAs showed structural changes consistent with the onset and progression of PVD, namely the extension of muscle into the walls of peripheral arteries and a reduction in arteries relative to alveoli. Other inflammatory agents, like tumor necrosis factor (TNF) -α, have also been reported to cause PH (Stevens, et al., 1992). In the next section, the toxin-induced model of PH, used in this study, will be discussed.
The Monocrotaline Model of Pulmonary Vascular Disease

Monocrotaline (MCT), a pyrrolizidine alkaloid, is derived from the seeds of Crotalaria spectabilis (Allen, 1963; Hayashi, et al., 1967). Human exposure to MCT-like compounds is most frequently in the form of herbal or medicinal teas, often inducing liver damage and veno-occlusive disease (Chesney and Allen, 1973). While involvement of the pulmonary vasculature has not been described in humans, the major target organ of MCT exhibits species variation. In rats and canines, MCT primarily produces pneumotoxicity and PH (Cheeke and Pierson-Goeger, 1983). The frequency, nature, and severity of vascular lesions, in response to MCT, varies with age, dosage, route of administration, duration of exposure (Goldenthal, et al., 1964; Lalich, 1964; Merkow and Kleinerman, 1966; Sugita, et al., 1983), as well as species and strain, such that some animals, like gerbils, hamsters and guinea pigs, are basically resistant (Cheeke and Pierson-Goeger, 1983). To exert its effects, MCT requires hepatic biotransformation, to become converted, via microsomal metabolism, to multiple reactive pyrroles that produce its observed organotoxic effects (Lafranconi and Huxtable, 1984; Lafranconi, et al., 1985; Dueker, et al., 1992). Both the mixed function oxidase (MFO) system and a cytochrome P-450 dependent pathway, specifically involving cytochrome P-450 3A (Reid, et al., 1998), in liver microsomes appear to be involved (Mattocks and White, 1971; Ramsdell, et al., 1987). Radio-tracer studies by Estep et al (Estep, et al., 1991), using $^{14}$C-MCT, show that while rapid elimination of radioactivity occurred through the urine and bile by 7 hours, high levels of the radioactive pyrroles metabolites were retained in red blood cells and disseminated from the liver to other organs including the lung. Mattocks (Mattocks, 1968) suggested that a property of this pyrrole as an electrophile, might permit covalent binding to certain tissue nucleophiles. Characterization of this pyrrole began with the observation that a novel Ehrlich-positive metabolite existed in the perfusate of a MCT-treated rat liver. Lafranconi (Lafranconi, et al., 1985) showed that this product could recapitulate the pneumotoxic action of MCT and Glowaz and coworkers (Glowaz, et al., 1992) determined the major reactive product
released from MCT-treated isolated rat liver microsomes to be 7-ethoxy-1-hydroxymethyl-6,7-dihydro-5H-pyrrolizine (O7-ethyldehydroretroene) by using the sulfhydryl-containing resin, thiopropyl sepharose 6B, as a trapping agent. Very recently, one study by Huan et al (Huan, et al., 1998) has shown that it is the difference in the ability to process and produce this reactive pyrrolizine that accounts for the variation in species susceptibility to MCT.

Administration of monocrotaline as a single large dose or chronically as a series of lower doses has been associated with hepatotoxicity (Bull, 1955; Schoental and Mattocks, 1960). Megalohepatocytosis, inhibition of liver cell mitosis with DNA damage (Petry and Sipes, 1987), and hepatocellular carcinoma have been reported in rats given a frequent low dose regime (Schoental, 1968; Hooson and Grasso, 1976). In addition, renal lesions including glomerular necrosis, hyaline thrombosis of the glomerular capillaries and afferent arterioles, and mesangiolysis have been reported (Carstens and Allen, 1970; Kurozumi, et al., 1983). The lung toxicity, for which we use this model, is associated with pulmonary arterial changes that can be correlated with hemodynamic evidence of progressive pulmonary hypertension and increased pulmonary vascular resistance (Plestina and Stoner, 1972; Hislop and Reid, 1974; Meyrick, et al., 1980; Todd, et al., 1985; Todorovich-Hunter, et al., 1988; Todorovich-Hunter, et al., 1992; Wilson, et al., 1992). After administration of MCT, a rise in cardiac output is initially observed by day 7 and is related to structural changes, specifically the extension of muscle in peripheral arteries. The muscularization of normally non-muscular PAs continues without a significant elevation in PA pressure until day 14. At this timepoint, an observable increase in medial wall thickness of normally muscular arteries is evident and these vessels become progressively more hypertrophied along with corresponding pressure increases. By day 21, pulmonary vascular resistance becomes elevated together with a reduction in cardiac output. Concomitantly, there is a loss of arterial concentration and pulmonary pressure continues to rise. At one month, pulmonary vascular
resistance is six times normal and together with marked right ventricular hypertrophy, congestive heart failure results.

Ultrastructural studies have shown injury to the vascular endothelium within several days of MCT-injection (Rosenberg and Rabinovitch, 1988; Todorovich-Hunter, et al., 1992). While the exact mechanism of injury is unknown, a loss of some endothelial cells by apoptosis is observed (Jones and Rabinovitch, 1996; Thomas, et al., 1998). As replacement of these cells is delayed, through the ability of MCT to inhibit endothelial cell proliferation (Lappin, et al., 1998), a persistent pulmonary vascular leak results in the development of subendothelial edema and inflammation which is apparent after one week. Endothelial dysfunction has been documented by Shubat et al (Shubat, et al., 1990) as dysregulation of membrane Na\(^{2+}/K^+\) pumps and by Olson (Olson, et al., 1984) as sustained activation of lung ornithine decarboxylase, both occurring rapidly following MCT-treatment. In studies targeting impaired endothelial-dependent relaxation (Mathew, et al., 1995) with either L-arginine (Mitani, et al., 1997), the precursor of NO, or with an endothelin A receptor antagonist (Prie, et al., 1998), pulmonary hypertension has been effectively reduced. Thus, the induction of vascular disease is likely mechanistically related to this endothelial injury.

In addition to alterations in endothelial function, abnormal metabolites of smooth muscle cells and macrophages may also contribute to the pathophysiology of monocrotaline-induced pulmonary hypertension. Arcot et al (Arcot, et al., 1995) have noted an early progressive increase in the expression of basic fibroblast growth factor (bFGF) and Stenmark and coworkers (Sugita, et al., 1983; Stenmark, et al., 1985) have reported the abnormal accumulation of large intra-alveolar macrophages as a very early feature of MCT-induced PH. To address the mechanism of macrophage infiltration and its role in the development of vascular disease, Kimura and colleagues (Kimura, et al., 1998) monitored for the presence of the potent monocyte chemotactic and activating chemokine/monocyte
chemoattractant protein-1 (MCAF/MCP-1) in the plasma of MCT-treated rats. Finding that MCAF/MCP-1 was elevated by day 7, these investigators began intravenous administration of anti-MCAF/MCP-1 antibodies into the rats and documented both a decrease in macrophage infiltration and reduced RVH and right ventricular systolic pressure, as well as less severe medial thickening of pulmonary arterioles. Additional strategies like the delivery of the interleukin-1 receptor antagonist have also been successful in limiting MCT-induced pulmonary vascular changes (Voelkel, et al., 1994).

Our laboratory compared the response of rats to MCT at various stages in lung development (Meyrick, et al., 1980; Todd, et al., 1985). Neonatal, infant and adult rats were injected with MCT and, while neonatal rats did not survive to the third week, owing to a severe impairment of alveolar development, similar vascular changes were evident in all three groups. The progressive increase in arterial muscularity and decrease in arterial number was observed by the second week and while these changes continued in the adult, infant rats exhibited a spontaneous regression during the second to fourth week. Ultrastructurally, the difference between the initiation of malignant pulmonary vascular changes in the adult rat versus the regressive phenotype exhibited by the infant rats was that the adult rats showed fragmentation of the internal elastic lamina (Todorovich-Hunter, et al., 1992). This degradation was evident by day four following injection and suggested the rapid activation of an enzyme that could proteolyze elastin and potentially other ECM components. Our laboratory then went on to show an early twofold rise in elastolytic activity on the second day after MCT as well as a second increase between day 16 and 28, that was absent in infant rats, and correlated with the progression of structural and functional vascular alterations in the adult animals.

The notion that this progressive elastolytic activity was related to the development of malignant abnormalities is consistent with the observation that reversible hypoxia-induced
hypertension is associated with only a transient rise in elastase activity (Maruyama, et al., 1991). Our laboratory has characterized the elastase enzyme to be a ~20kD serine proteinase, related to the serine proteinase adipsin, and localized to the vascular SMCs (Zhu, et al., 1994). Indeed, serine elastase inhibitors, given for one week beginning at the time of MCT-injection, suppress the rise in elastolytic activity and reduce the sequelae of vascular changes in response to MCT-injection (Ilkiw, et al., 1989; Shemie and Rabinovitch, 1993). Interestingly, elastase inhibition also protected the endothelium from MCT probably by preventing the rise in pulmonary artery pressure, which contributes to progressive subendothelial edema (Ye and Rabinovitch, 1991). Delayed delivery of the elastase inhibitor was also effective in reducing pulmonary vascular remodeling (Ye and Rabinovitch, 1991).

III. Role of Serine Elastases and Matrix Metalloproteinases in Vascular Pathobiology

Serine Elastases

Elastases can be serine, cysteine, or metallo proteinases with the ability to solubilize elastin fibres as well as other extracellular matrix proteins (Bieth, 1978; Bieth, 1989). Serine elastases have been extensively studied and have been linked to the development of pulmonary hypertension (Todorovich-Hunter, et al., 1992; Zhu, et al., 1994) and thus will be the focus of this discussion. The substrate specificity of an elastase is determined by the molecular structure of its substrate binding and catalytic site. Serine elastases are defined by the serine, histidine, and aspartate amino acid hydrogen bonded together to form the catalytic triad of their catalytic site (Stryer, 1988). Hydrolytic activity of a serine elastase involves acylation, formation of a covalent acyl-enzyme intermediate, and deacylation which proceeds following the formation of the enzyme-substrate complex. Complex formation is achieved through the creation of a tetrahedral transition state between the reactive oxygen of the enzyme’s reactive serine and the carbonyl group of the substrate (Bieth, 1989).
Elastases are commonly expressed in tissues that exhibit a regular turnover of their extracellular matrix. Such tissues include the uterus during involution and the lung and vasculature during development. In these tissues, the activity of serine elastases are kept in check by the concomitant production of native inhibitors like α₁-proteinase inhibitor and α₂-macroglobulin (Salvesen and Travis, 1989). However, in association with a variety of vascular diseases, a proteinase-anti-proteinase imbalance occurs, with an excess of serine elastolytic activity being related to disease pathogenesis (Oho and Rabinovitch, 1994; Oho, et al., 1995; Blann, et al., 1996; Mohacsi, et al., 1996; Robert, et al., 1998; Rao, et al., 1999). For example, increased elastolytic activity has been associated with the formation and progression of atherosclerotic lesions (Hornebeck, et al., 1978; O'Brien and Regan, 1991). Our group has shown, in porcine aortic organ culture that neointimal formation was related to increased elastase activity (Oho, et al., 1995). That is, inhibition of serine elastases with α₁-proteinase inhibitor reduced the recruitment of SMCs to the neointima. While serine elastases can be produced locally by vascular smooth muscle cells as we (Kobayashi, et al., 1994; Zhu, et al., 1994) and others (Bourdillon, et al., 1984; Hornebeck, et al., 1985) have described, potent serine elastases such as human leukocyte elastase, released by neutrophils, have been causally related to endothelial cell damage in the development of peripheral atherosclerosis (Blann, et al., 1996; Mohacsi, et al., 1996).

Consistent with reports in intracranial and cerebral aneurysms (Baker, et al., 1995; Marshman, 1998), Rao and colleagues (Rao, et al., 1999) have described an elastase/anti-elastase imbalance in the pathogenesis and rupture of abdominal aortic aneurysms. The mechanism was investigated by Wills et al (Wills, et al., 1996), who concluded that the heightened serine elastase activity orchestrated matrix degradation, through the production and activation of MMPs-1, -2, -3, and -9, leading to weakening and rupture of the vessel wall. This study suggested that serine elastases may be able to promote more extensive
ECM remodeling through the recruitment and proteolytic activation of MMPs. Our laboratory has described a role for a 23 kDa serine elastase in the pathogenesis of the post-cardiac transplant coronary arteriopathy (Oho and Rabinovitch, 1994) associated with ultrastructural observations of fragmented elastic laminae (Billingham, 1992). The serine elastolytic activity measured in donor coronary arteries following heterotopic heart transplant in piglets may be partly attributable to SMCs as well as to the infiltration and release of neutrophil elastase by inflammatory cells, T-lymphocytes and macrophages. More recent studies have supported a role for this elevated coronary artery elastase activity in the modulation of fibronectin synthesis, another feature described in allograft arteriopathy (Clausell, et al., 1993a). Elastin peptides, the product of elastase-mediated elastin proteolysis, can induce an increase in SMC fibronectin synthesis by a post-transcriptional mechanism (Cowan, B. et al. 2000, submitted manuscript) (Hinek, et al., 1996). Indeed, the use of the serine elastase inhibitor, elafin, abrogates the increased coronary artery fibronectin synthesis and the development of the post-cardiac transplant arteriopathy in experimental animals (Cowan, et al., 1997). This suggests that elastase-mediated fibronectin expression is an important step in the formation of neointimal lesions in occlusive systemic vascular diseases.

Our laboratory recently made a transgenic mouse that overexpresses the serine elastase inhibitor elafin targeted to the cardiovascular system by the preproendothelin promoter. This permitted us to explore the importance of proteinase/anti-proteinase balance in the progression of cardiovascular disease (Zaidi, et al., 1999a). Using a model of carotid artery wire injury, the transgenic mice with heightened elastase-inhibitory activity, when compared to their non-transgenic littermates, showed reduced intimal formation associated with a decreased inflammatory cell infiltrate and absence of an upregulation of the SMC pro-proliferative ECM glycoprotein, tenascin-C (Zaidi, et al., 1999b). Similarly, we have shown that during the arterialization of vein grafts in rabbits, transfection with an expression vector
encoding the elastase inhibitor, elafin, resulted in reduced intimal formation and atherosclerotic degeneration related to suppression of tenascin-C and vascular smooth muscle proliferation, and a reduction in macrophages and T-cells (O'Blenes, et al., 1998). This reinforces the fact that, in response to injury, heightened elastase activity promotes vascular cell proliferation as well as recruitment of inflammatory cells.

As previously described, we have related elevated serine elastase activity to the initiation of PH following exposure to hypoxia or MCT (Maruyama, et al., 1991; Todorovich-Hunter, et al., 1992), and to the malignant progression of functional and structural abnormalities in the pulmonary vasculature after administration of MCT to rats (Todorovich-Hunter, et al., 1992). As both elastase activity and endothelial cell injury are the earliest features following the MCT insult, and as endothelial damage is thought to be the target of MCT, our laboratory has investigated how endothelial cell abnormalities might be related to induction of serine elastases by vascular SMCs. The development of edema in response to MCT suggested that the structural and functional alterations in the vascular endothelial cells resulted in a loss of barrier properties. Our laboratory hypothesized that as a result of this leaky barrier, serum factors would penetrate into the subendothelium and interact with the resident SMCs to induce elastase activity. In early assays, PA or aorta-derived SMCs, cultured with radiolabeled elastin, would only degrade the elastin in the presence of serum. With elastase inhibitors in serum, the activity was thought to be localized in the pericellular region and, correspondingly, was associated with increased elastin adhesion to the 67 kDa elastin binding protein on SMC surfaces (Hinek, et al., 1988; Kobayashi, et al., 1994).

This elastase activity could be recapitulated by elastin pre-treated with serum or pretreatment of the SMCs with serum confirming that the serum factor required tethering to the SMC surface (Thompson, et al., 1998). A 27 kDa serum protein was enriched by elastin affinity chromatography and N-terminal microsequence analysis suggested that it was
apolipoprotein Al. Indeed, purified apolipoprotein Al was able to induce smooth muscle cell elastase activity. Following binding of elastin to SMCs via serum factors, such as apolipoprotein Al, an intracellular tyrosine kinase cascade was activated, associated with phosphorylation of focal adhesion kinase (FAK) and MAP (mitogen-activated protein) kinase members, including ERK-1/2 (extracellular signal related kinase). As the induction of elastase activity required both mRNA transcription and protein translation, mRNA differential display was used to identify transcripts expressed coincident with elastase induction (Wigle, et al., 1998). A transcript homologous to the transcription factor AML (acute myelogenous leukemia) -1 was identified. Since AML-1 is a transcription factor for neutrophil elastase (Nuchprayoon, et al., 1994), it also is likely to be the transcription factor for the vascular elastase. To support this, AML-1 protein is elevated in the nucleus of SMCs in association with serum-induced elastase activity and its nuclear expression is regulated by MAP kinase activity. Moreover, AML-1 antisense oligonucleotides effectively repressed serum-treated elastin induction of elastase activity. This pathway, therefore, suggests a causal link between monocrotaline-induced endothelial cell damage and induction of serine elastase activity. Interestingly, NO inhibits ERK-1 phosphorylation, through a protein kinase G (PKG) dependent pathway, and this suppresses nuclear expression of AML-1 and elastase activity (Mitani, Y. et al 2000, in press). This may suggest that the suppression of NO production in hypertensive arteries may contribute to heightened elastase activity.

The sequelae of induction of elastase can be related to the pathophysiology of pulmonary vascular disease. The proteolysis of elastin, by elastases, is associated with the liberation of matrix-bound SMC growth factors, like bFGF (Thompson and Rabinovitch, 1996), and potentially other growth factors like transforming growth factor (TGF) -β, which is preferentially released from matrix by serine proteinase degradation (Taipale, et al., 1995). While these growth factors may stimulate SMC proliferation directly, bFGF and TGF-β can
also upregulate the production of extracellular matrix proteins, elastin (Liu and Davidson, 1988), collagen (Neubauer, et al., 1999), and the glycoprotein, tenascin-C (Rettig, et al., 1994; Mackie, et al., 1998), which specifically amplifies the proliferative response of SMCs (Jones and Rabinovitch, 1996; Jones, et al., 1997b). It is of further interest that, the downstream effects of elastase upregulation might also include increased expression and deposition of fibronectin, through the degradation of elastin and production of elastin-derived peptides (Cowan et al 2000, revised manuscript). This fibronectin, which has been shown to be pro-migratory for SMCs during neointimal formation (Boudreau, et al., 1991; Molossi, et al., 1995b), may contribute to pulmonary hypertensive lesions by facilitating a directed migration of SMCs into the subendothelial space. This suggests that elastase may be capable of orchestrating the formation of pulmonary vascular lesions (Fig. 0.2).

**Matrix Metalloproteinases**

The family of enzymes called matrix metalloproteinases, which include the collagenases, stromelysins and gelatinases, are a structurally related group of zinc and calcium dependent matrix proteinases (Murphy, et al., 1991; Woessner, 1991). Under normal physiological conditions, MMPs are involved in vital tissue remodeling processes such as wound healing, pregnancy, and vasculogenesis. Matrix metalloproteinases are activated from a pro-enzyme or latent form either enzymatically or by mercurial agents or heat (Okada and Nakanishi, 1989; Knauper, et al., 1990; Nagase, et al., 1990; Koklitis, et al., 1991). They are also controlled by endogenous inhibitors, e.g., the tissue inhibitors of metalloproteinases (TIMPs) (Cawston, et al., 1981; Greene, et al., 1996) which form 1:1 covalent complexes with MMPs (Gomis-Ruth, et al., 1996). Matrix metalloproteinases, as a family of proteinases, can degrade most ECM components (Carmeliet and Collen, 1998). In pathological states, the excessive production and/or activation of MMPs leads to accelerated
Figure 0.2

Schematic summary of the putative mechanism involved in the initiation and progression of pulmonary vascular disease, as described in the text.
Endothelium

elastin lamina

injury

Serum leak

ELASTASE (EVE)

MMPs

SMC PROLIFERATION

HYPERTROPHY, CT SYNTHESIS

bFGF TGFβ release

Tenascin

Fibronectin

elastin peptides

SMC MIGRATION
matrix degradation. Substrate cleavage by MMPs occurs at a catalytic site containing several calcium atoms and two zinc atoms that are chelated by histidine residues, one of which forms the active site of the enzyme. Together with the catalytic site, which determines enzyme activity, macromolecular substrate recognition is believed to occur through the C-terminal domain (Allan, et al., 1991; Murphy and Hembry, 1992; Hirose, et al., 1993; Nagase and Fields, 1996).

The role of matrix metalloproteinases in vascular pathologies has been well documented (Ross, 1993; Dollery, et al., 1995; Libby, 1995; Celentano and Frishman, 1997). In the development of atherosclerosis, evidence indicates that increases in reactive oxygen species and urokinase plasminogen activator upregulate MMPs in vulnerable regions of atherosclerotic plaques leading to plaque instability (Galis, et al., 1994; Brown, et al., 1995a; Halpert, et al., 1996; Rajagopalan, et al., 1996; Carmeliet, et al., 1997). Galis and coworkers (Galis, et al., 1994) identified MMPs-1, -2, -3, and -9 as being upregulated by both lipid-laden macrophages and vascular SMCs (Galis, et al., 1995a; Galis, et al., 1995b), and suggest that these MMPs may function to promote destabilization and complication of atherosclerotic plaques. Indeed, investigations by Brown and colleagues (Brown, et al., 1995a) implicated MMP-9 as pivotal to human coronary atherosclerotic plaque rupture and associated acute coronary ischemia. Matrix metalloproteinase (MMPs-1, -2, -3, and -9) mediated proteolysis also appears to contribute to the ulceration and rupture of aneurysms (Newman, et al., 1994; Patel, et al., 1996; Carmeliet, et al., 1997; Knox, et al., 1997).

The major complication in using percutaneous transluminal coronary angioplasty to treat patients with atherosclerotic coronary disease is the frequency of restenosis (30% in some series) (Boyle, et al., 1998). Strauss and colleagues have shown that experimentally-induced restenotic lesions are characterized by MMP activity and excessive deposition of ECM components (Strauss, et al., 1994; Strauss, et al., 1996). Heightened activities of
MMPs-2 and -9 have been identified following balloon injury (Zempo, et al., 1994; Tyagi, et al., 1995) and MMP inhibition has resulted in partial reduction in lesion size due to both a decrease in ECM accumulation and an inhibition of vascular SMC migration (Strauss, et al., 1996; Zempo, et al., 1996; Dollery, et al., 1999). These studies suggest that MMPs could stimulate both ECM synthesis and SMC migration.

Matrix metalloproteinases are also critical in endothelial cell motility associated with tumour angiogenesis. Indeed, the ability to block angiogenesis with MMP inhibitors demonstrates their mechanistic importance in this process (Galardy, et al., 1994; Wojtowicz-Praga, et al., 1997). The switch to an invasive endothelial cell phenotype is associated with the expression of MMP-2 and MT1-MMP (membrane type matrix metalloproteinase) among other MMPs (Sato, et al., 1997). While MT1-MMP expression has been related to the activation of a proteolytic cascade involving the recruitment and activation of MMP-9 and MMP-13 (Cowell, et al., 1998), the binding and activation of MMP-2 to MT1-MMP as well as to αvβ3 integrins has been suggested to target matrix degradation to the leading edge of the endothelial cell (Brooks, et al., 1996; Haas and Madri, 1999).

Little is known about the role of MMPs in the development of pulmonary hypertension. However, given the existence of elevated serine elastase activity, it is likely that MMPs in their inactive form could become proteolytically activated (Fig. 0.3) (Okada and Nakanishi, 1989; Nagase, et al., 1990; Matsumoto, et al., 1992). In addition, elastases have been reported to degrade TIMPs, including those in active complex with MMPs (Okada, et al., 1988; Itoh and Nagase, 1995). Through the production of elastin-derived peptides and release of growth factors, like bFGF, elastases may induce an upregulation of MMP activity (Landeau, et al., 1994; Aho, et al., 1997; Miyake, et al., 1997) during the pathogenesis of
Figure 0.3

Schematic of potential pathways by which serine elastases may regulate matrix metalloproteinase (MMP)-2, as described in the text. Briefly, elastases may regulate MMP-2 expression through extracellular matrix proteolysis which release growth factors, like basic fibroblast growth factor and transforming growth factor beta, and extracellular matrix fragments, such as elastin, collagen and fibronectin derived peptides, all of which can regulate transcription of MMP-2. Elastases have also been reported to proteolytically activate latent MMP-2 which could occur directly or through elastase-mediated presentation of MMP-2 to membrane type 1-MMP (MT1-MMP). In addition, degradation of tissue inhibitors of metalloproteinases (TIMPs) by elastases may further promote MMP activity. However, loss of TIMPs may also indirectly reduce MMP-2 activation by MT1-MMP which reportedly can occur through TIMP-2/MMP-2 complex binding to MT1-MMP. It is conceivable that TIMP-2-elastase complexes facilitate TIMP-2/MMP-2 binding to MT1-MMP, but this has not been described.
pulmonary vascular lesion formation. Once activated, MMPs can degrade native inhibitors of elastase such as alpha1-proteinase inhibitor (Kataoka, et al., 1999). This suggests that these two enzyme systems, once activated, may function in a codependent fashion. Our laboratory has reported that pulmonary artery SMCs in culture on collagen gels produce excessive MMP-2 (Jones, et al., 1997b). Through MMP-mediated degradation of type I collagen, cryptic RGD (arginine-glycine-aspartic acid) motifs are exposed (Davis, 1992). Ligation of the PA SMCs to this degraded collagen occurs preferentially through β3 containing integrins. β3 integrin ligation activates the MAP kinases, ERK-1/2, which target the morphoregulatory paired-related homeobox transcription factors, prx-1 and prx-2, to bind and activate an extracellular matrix responsive element in the tenascin-C gene promoter (Jones, et al., 1999). Subsequently, tenascin-C transcription and deposition is associated with a growth response in the SMC population (Fig. 0.4). Conversely, inhibition of MMPs, with specific inhibitors or under ‘floating’ collagen gel conditions, is associated with a reduction in tenascin-C expression. These observations are in keeping with reports that both active MMPs and tenascin-C are present at sites of vascular remodeling (Hedin, et al., 1991; Mackie, et al., 1992; Zempo, et al., 1994; Strauss, et al., 1996). These features suggest that, in response to increased elastase activity, MMPs may be activated in pulmonary vessels and, through matrix degradation, lead to the upregulation of tenascin-C and increased SMC proliferation, as observed in the MCT model. Recent studies examining the regression of hypoxia-induced pulmonary vascular abnormalities following the return of experimental rats to normoxia have reported an increase in both vascular and mast cell-derived MMPs (Thakker, et al., 1998; Tozzi, et al., 1998). While this suggests that such MMP activity may be pivotal to the regression process, a cause and effect relationship has not yet been established.
Figure 0.4

**Hypothetical model for the regulation and function of tenascin-C in vascular smooth muscle cells.** (A) Vascular smooth muscle cells (SMCs) attach and spread on native type I collagen using β1 integrins. Under serum-free conditions, the cells withdraw from the cell cycle and become quiescent. (B) Degradation of native type I collagen by matrix metalloproteinases (MMPs) leads to the exposure of cryptic RGD sites that preferentially bind β3 subunit-containing integrins. In turn, occupancy and activation of β3 integrins signal the production of tenascin-C (TN). (C) Incorporation of multivalent TN protein into the underlying substrate leads to further aggregation and activation of β3-containing integrins (αvβ3), and to the accumulation of tyrosine-phosphorylated (Try-P) signaling molecules and actin into a focal adhesion complex (FAC). Note that even in the absence of the epidermal growth factor (EGF) ligand, the TN-dependent reorganization of the cytoskeleton leads to clustering of actin-associated EGR receptors (EGR-Rs). (D) Addition of EGF ligand to clustered EGF-Rs results in rapid and substantial tyrosine phosphorylation of the EGF-R and activation of downstream pathways culminating in the generation of nuclear signals leading to cell proliferation. (From Jones, P.L. et al. *J. Cell Biol.* 1997; 139: 279-293.)
IV. Tenascin-C and Osteopontin as Vascular Cell Survival Factors

Tenascin-C as a Vascular Cell Survival Factor

Our laboratory has implicated tenascin-C in the progression of pulmonary vascular disease. This large ECM glycoprotein is a member of a family of four highly conserved proteins encoded by different genes (tenascin-C, -R, -X, and -Y) (Mackie, 1997). These proteins contain a number of epidermal growth factor (EGF) like domains, a series of fibronectin (FN) type III repeat domains, a carboxy terminal domain homologous to the beta and gamma chains of fibrinogen, and a ‘central’ domain in which monomers form disulfide bonds creating tenascin’s characteristic hexabrachion structure (Fig. 0.5) (Erickson and Inglesias, 1984; Erickson and Taylor, 1987; Jones, et al., 1988). Tenascin-C interacts with cells as well as other ECM components (Jones, et al., 1989; Spring, et al., 1989), although with variable binding affinities related to the different isoforms (products of alternatively spliced mRNA transcripts).

Cellular interactions with tenascin-C occur via integrin receptors (including the αvβ3), contactin/F11 (adhesion molecule of the Ig superfamily), annexin II, a receptor tyrosine phosphatase and cell surface heparan sulphate proteoglycans (Zisch, et al., 1992; Aukhil, et al., 1993; Sriramarao, et al., 1993; Barnea, et al., 1994; Chung and Erickson, 1994). Tenascin-C is expressed during development in mesenchymal tissues and is particularly prominent in the skin, brain, connective tissue and cartilage (Erickson, 1993). In the adult, however, tenascin-C is only expressed during tissue restructuring related to tissue involution and pathological processes including wound healing, and cancer (Chiquet-Ehrismann, et al., 1986; Mackie, et al., 1988; Koukoulis, et al., 1993; Boudreau, et al., 1996). In these processes and in cell culture systems, tenascin-C has been associated with migration, proliferation, differentiation, apoptosis and loss of tissue-specific gene expression.
Figure 0.5

Schematic of the modular extracellular matrix glycoprotein tenascin-C.
Tenascin-C
Recently, tenascin-C has been described in arterial remodeling associated with balloon catheter injury (Hedin, et al., 1991; Majesky, 1994), the development of monocrotaline-induced pulmonary hypertension (Jones and Rabinovitch, 1996; Lipke, et al., 1996), the arterialization of vein grafts (O'Blenes, et al., 1998; Wallner, et al., 1999a) and formation of atherosclerotic plaques (Wallner, et al., 1999b).

In normal rat carotid arteries, tenascin-C mRNA is barely detectable. However, within 6 hours following injury, tenascin-C becomes highly expressed (Majesky, 1994). The heightened expression period, which lasts for approximately two weeks, correlates with the formation of a neointima involving the proliferation and migration of SMCs. Similarly, in the malignant form of pulmonary vascular disease in the adult rat following monocrotaline injection, early endothelial cell apoptosis precedes tenascin-C deposition and is temporally associated with smooth muscle cell proliferation (Jones and Rabinovitch, 1996). In contrast, tenascin-C upregulation is not apparent in the infant rat in which monocrotaline-induced pulmonary hypertension spontaneously reverses.

The mechanism relating tenascin-C expression with smooth muscle cell proliferation was delineated in cell culture. Chiquet-Ehrismann (Chiquet-Ehrismann, et al., 1994) had demonstrated that on attached collagen gels, chick embryo fibroblasts express tenascin-C whereas on floating gels, tenascin-C is preferentially suppressed. Our group showed that rat pulmonary artery SMCs respond similarly to changes in shape using this system (Fig. 0.6) (Jones, et al., 1997b). It is of further interest that microarray technology identified tenascin-C as a gene upregulated in SMCs exposed to cyclic strain (Feng, et al., 1999). On attached collagen, deposition of endogenous tenascin-C is enhanced and acts as a pro-proliferative factor in that it further increases SMC number in response to FGF-2 and is a
Figure 0.6

Schematic representation of pulmonary artery smooth muscle cells on attached and floating collagen gels and, the effects on tenascin-C production. The blot represents an autoradiograph showing immunoprecipitated tenascin-C protein from [\(^{35}\)S]methionine/cysteine-labeled smooth muscle cell lysates harvested from attached and floating collagen gels at 24 hours. Synthesis of tenascin-C protein isoforms, of apparent molecular masses 220 and a doublet at 180 kDa, is suppressed in cells cultured on floating collagen. (From Jones, P.L. et al. J. Cell Biol. 1997; 139: 279-293.)
Attached Cultures

Floating Cultures

Attached Floating

220 kDa TN-C

180 kDa TN-C
prerequisite for EGF-dependent SMC proliferation. The mechanism by which proliferation is induced in response to tenascin-C involves a change in cell shape. While some cell types exhibit a rounded cell morphology in the presence of tenascin-C (Spring, et al., 1989; Prieto, et al., 1992; Crossin, 1994), vascular SMCs as well as endothelial cells spread (Sriramarao, et al., 1993; Jones and Rabinovitch, 1996). The process is related to a rearrangement of the filamentous actin cytoskeleton induced by ligation of cell surface integrin receptors to tenascin-C in the extracellular matrix (Fig. 0.3). Similar to endothelial cells (Prieto, et al., 1993; Sriramarao, et al., 1993), SMC interaction with tenascin-C is mediated by the αvβ3 integrin receptor. The clustering of integrins leads to rearrangement of actin filaments into focal contacts and concomitant aggregation of growth factor receptors (Jones, et al., 1997b). Specifically, EGF receptors are clustered and efficient receptor phosphorylation is evident following ligation with EGF, leading to a tyrosine phosphorylation cascade and a nuclear signal associated with cell growth. The collaboration of TN with growth factor receptors to induce proliferation has also been reported in other cell types (End, et al., 1992; Chung and Erickson, 1994; Chung, et al., 1996).

As the use of a distinct repertoire of receptors by SMCs maintains their contractile and quiescent phenotype (Thyberg, 1996), the use of different integrin receptors signal changes in cell shape, which lead to altered gene expression and cellular function (Folkman and Moscona, 1978; Ingber, et al., 1995; Chen, et al., 1997) necessary for SMC survival during vascular remodeling. In endothelial cells, the switch to an angiogenic phenotype in response to bFGF is associated with increased expression and usage of the αvβ3 integrin (Boudreau, et al., 1997). Following establishment of the 'angiogenic' phenotype, selective signaling through the αvβ3 integrin suppresses both p53 activity and p21WAF1/CIP1 expression, increases the bcl-2/bax ratio and sustains ERK activity (Stromblad, et al., 1996; Eliceiri, et al., 1998). On the other hand, αvβ3 antagonists induce apoptosis in remodeling, but not in preexisting normal vessels (Brooks, et al., 1994a). This is consistent with the transient

Using attached versus floating collagen gels, our group observed that rat PA SMCs on floating collagen, in which endogenous tenasin-C is repressed, undergo apoptosis (Jones and Rabinovitch, 1996). The importance of tenasin-C withdrawal in this process is underscored by studies in which addition of exogenous tenasin-C rescues the SMCs from apoptosis (Fig. 0.7). Consistent with these cell culture findings, recent studies of vascular thickening following balloon catheter injury, have also shown elevated expression of tenasin-C during neointimal development (Hedin, et al., 1991; Majesky, 1994), and the efficacy of an αvβ3 functional blocking antibody administered at the time of injury in inducing apoptosis and subsequent reduction in intimal thickening (van der Zee, et al., 1998). While tenasin-induced survival involves signaling through clustered β3 integrins, the mechanism of apoptosis following blockade of αvβ3 receptors remains to be determined.

**Could Alternative β3 Integrin Ligands Replace Tenascin-C?**

Despite evidence supporting a critical role for tenasin-C in vascular cell survival, a tenasin-C knockout mouse was created which was not embryonically lethal and showed normal development (Saga, et al., 1992). In fact, even following very comprehensive examination of this mouse, only a subtle phenotype was evident (Fukamauchi, et al., 1996). This phenotype, characterized by hyperlocomotion, poor swimming ability, and inability to adjust to new environments, has been attributed to an interruption of dopamine signaling within the developing central nervous system (Fukamauchi and Kusakabe, 1997; Fukamauchi, et al., 1997), where tenasin-C is abundantly expressed. Considering the critical survival function
Figure 0.7

Tenascin-C acts as a vascular smooth muscle cell survival factor. A, Effect of exogenous tenascin-C on smooth muscle cell numbers on floating collagen gels. A significant decline in smooth muscle cell numbers is apparent on floating compared to attached collagen gels (p<0.05), whereas addition of tenascin-C suppresses this effect. B, Effect of tenascin-C on smooth muscle cell apoptosis on floating collagen gels. Smooth muscle cells plated on attached collagen were maintained for 48 hours or were floated in the same medium, either with or without addition of exogenous human tenascin-C (15 mg/ml). Genomic DNA was isolated from each culture and 10μg per sample were analyzed on agarose gels. DNA fragments comprised of ~180-bp multimers, which are indicative of apoptosis, were apparent on floating collagen gels. In contrast, no evidence of DNA fragmentation was observed on either attached or tenascin-supplemented floating collagen. (From Jones, P.L. et al. J. Cell Biol. 1997; 139: 279-293.)
Figure A shows a bar graph comparing cell number \( \times 10^3 \) at 48 h between two conditions: Att and Floating. The graph includes two bars, one for Collagen and another for Collagen + TN-C. The Collagen bar shows a higher cell number compared to Collagen + TN-C.

Figure B presents a comparison between Att and Floating conditions with Collagen and Collagen + TN-C treatments. The graph indicates a distribution of values at 0.5, 1.0, 1.6, 2.0, and 3.0.
of tenascin-C and the lack of an impressive embryonic phenotype in knockout mice, there are likely additional proteins that are αvβ3 ligands, and that could replace tenascin-C and signal survival. Indeed the αvβ3 integrin receptor is a promiscuous receptor (Byzova, et al., 1998) with numerous ligands including multiple ECM proteins, particularly the ECM phosphoprotein osteopontin recently reported to have vascular cell survival functions.

Osteopontin is an acidic phosphorylated glycoprotein identified initially in bone (Denhardt and Guo, 1993). This extracellular matrix protein is fairly unique in that it does not contain a modular domain structure. However, it does contain a sequence with some homology to a heparin-like binding domain, a serine-X-glutamine rich repeat sequence that is likely involved in O-linked oligosaccharide conjugation, and a GRGDS integrin recognition domain that shows the highest homology across species. Osteopontin is expressed in a tissue-specific fashion, being regulated by numerous factors including hormones, growth factors like bFGF, and cytokines like TGF-β (Ramos, 1999), and it is believed to exist as isoforms, related to alternative splicing of its mRNA transcript (Ullrich, et al., 1991). Osteopontin is normally expressed in tissues such as bone and kidney as well as along the luminal surface of most epithelial cell layers (Brown, et al., 1992). A marked upregulation of osteopontin has been documented in cancerous tissues (Brown, et al., 1994) and in vascular pathology (O'Brien, et al., 1994).

Osteopontin is upregulated in experimental models of atherosclerosis (Parrish and Ramos, 1995) and in primary and restenotic atherosclerotic plaque tissue samples from patients (Giachelli, et al., 1993; O'Brien, et al., 1994). The source of osteopontin production in these plaques has been attributed to endothelial cells, SMCs and macrophages (O'Brien, et al., 1994) and osteopontin may contribute to the dystrophic calcification process observed in these lesions and to plaque instability, as speculated by Shanahan (Shanahan, et al., 1994). Balloon injury in the rat carotid artery was also associated with elevated osteopontin
expression correlated both temporally and spatially with phenotypically altered proliferating and migratory SMCs (Giachelli, et al., 1993). In these studies, upregulation of osteopontin was related to the induction by the transcription factor upstream stimulatory factor (USF) -1 within 24 hours of the vascular injury (Malyankar, et al., 1999). In a model of mesenteric arterial injury related to continuous infusion of angiotensin II, a spatial pattern of osteopontin expression was observed (Wiener, et al., 1996). The smallest arteries, which undergo structural remodeling associated with thickening, expressed high levels of osteopontin, whereas no induction of osteopontin was evident in the larger arteries which fail to thicken.

These studies suggest that osteopontin may be important to the development of functional alterations in SMCs during disease. In tissue culture, osteopontin induces endothelial and SMC spreading via αvβ3 integrin ligation and subsequent formation of focal adhesion contacts (Liaw, et al., 1994). As adhesion is critical for survival, Scatena and coworkers (Scatena, et al., 1998) explored the possibility that osteopontin induces a critical survival signal for endothelial cells. When cultured on osteopontin, endothelial cells were resistant to serum deprivation-induced apoptosis and this could be reversed with specific αvβ3 neutralizing antibodies. The mechanism of αvβ3 induced survival was related to sustained activation of nuclear factor (NF) -κB transcriptional activity by Ras and Src kinase signaling. In addition, Brand et al (Brand, et al., 1996) have detected activated NF-κB in vascular cells of atherosclerotic plaques, where its activation might be related to the induction of inhibitor of apoptosis protein (IAP) -1 which appears to directly bind to and inactivate caspases (Deveraux, et al., 1997; Erl, et al., 1999). These studies provide evidence that osteopontin, like tenascin-C, signals through the αvβ3 integrin to sustain vascular cell viability by preventing apoptosis.
V. Apoptosis in Vascular Pathology

Apoptosis is a process of cell death that was initially described by its morphological characteristics. These features include cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation (Kerr, et al., 1994). Apoptosis is induced by some cell surface sensor, which invokes the intracellular signal transduction machinery and activates a cell death pathway. This process may be cell specific in the requirements for a selective set of cues to induce apoptosis.

Apoptosis is a process of critical importance in embryonic development and tissue homeostasis (Evan and Littlewood, 1998). In the cardiovascular system, the highly coordinated and spatially restricted timely loss of cells by apoptosis is thought to be responsible for the formation of the septum and valves in the heart and in the formation of vascular structures (Pexieder, 1975). Indeed, Cho and coworkers (Cho, et al., 1995) have reported increased apoptotic rates of endothelial cells and SMCs in the abdominal aorta and the intra-abdominal umbilical artery in lambs at parturition. These investigators related the onset of apoptosis to a necessary restructuring of the arterial system, in response to loss of blood flow through the umbilical arteries, and suggested that this was part of a more extensive process of apoptosis-mediated arterial reshaping during development.

In vascular pathology, apoptosis has been observed in primary atherosclerotic lesions (Han, et al., 1995; Isner, et al., 1995), in restenosis after percutaneous atherectomy (Isner, et al., 1995), and in saphenous vein aorto-coronary grafts following occlusion (Kockx, et al., 1994). In the rat model of vascular balloon catheter injury, two waves of apoptotic SMCs are observed. Clowes described that, as a result of the injurious insult, SMC proliferation and migration ultimately lead to the formation of a neointimal lesion which undergoes re-endothelialization (Clowes, et al., 1983). This process, which is associated with a net loss in
SMC number (Kocher, et al., 1984), involves a rapid induction of SMC apoptosis within the first 30 minutes after injury (Perlman, et al., 1997). A temporal overlap of proliferating and apoptotic SMCs is later observed especially in the population closest to the lumen, peaking at 20 days after balloon injury and ceasing with the completion of re-endothelialization of the vessel at about 45 days (Clowes, et al., 1983; Bochaton-Piallat, et al., 1995b; Han, et al., 1995). Low levels of apoptosis, observed in conjunction with continued cell proliferation, create a chronic remodeling process (Clowes, et al., 1983). The temporal co-existence of apoptotic and proliferating SMCs also suggests an equilibrium, which limits the extent of lesion formation.

In clinical disease, widespread apoptosis has been reported and attributed to both SMCs as well as lipid-laden macrophages (Kockx, et al., 1994; Geng and Libby, 1995; Han, et al., 1995; Isner, et al., 1995; Bjorkerud and Bjorkerud, 1996). Studies have indicated that in cultured SMCs from atherosclerotic lesions compared to SMCs from normal vessels, there is an increased susceptibility to apoptosis (Bennett, et al., 1995a; Bennett, et al., 1997) associated with heightened expression of the Fas death receptor (Geng, et al., 1997) and enhanced sensitivity to induction by oxidized LDL (Dimmeler, et al., 1997).

The localization of apoptotic cells towards the shoulder regions of the plaque (Geng and Libby, 1995) suggests that this feature might be associated with plaque instability, causing unstable angina pectoris and increasing the risk of acute myocardial infarction (Bennett and Boyle, 1998). Moreover, the presence of apoptotic endothelial and SMCs along the lesion cap may promote thrombus formation, since these cells can act as a substrate for thrombin (Bombeli, et al., 1997; Flynn, et al., 1997). Consistent with these speculations, studies by Bauriedel (Bauriedel, et al., 1997) have shown that higher apoptotic rates are correlated with unstable angina in patients. Nevertheless, it remains unclear as to whether apoptosis in
vascular pathologies serves as a counter-measure, limiting lesion formation, or contributes to the symptomatic presentation of the disease, or both.

**Apoptosis as a Potential Therapeutic Strategy**

The ability to control vascular smooth muscle cell apoptosis may offer tremendous potential to alter the structure and properties of the vessel wall. It is therefore necessary to know whether or not induction of apoptosis would be detrimental or beneficial, i.e., exacerbates or ameliorates lesion formation or progression. In addition, highly selective targeting of apoptosis would be a requirement of such therapy. In a recent study, deBlois and colleagues (deBlois, *et al.*, 1997) examined the effect of antihypertensive drugs on the lowering of blood pressure and associated effects on vascular remodeling in spontaneously hypertensive rats (SHR). The investigators noted that regression of vascular abnormalities was initiated by drugs that induced a vascular SMC apoptotic response. Such a response was the target of Steg and coworkers (Steg, *et al.*, 1997). Having observed that adenovirus-mediated overexpression of thymidine kinase in the presence of ganciclovir resulted in SMC apoptosis, these investigators performed angioplasty on rabbits with an adenovirus-coated balloon and, following peripheral injections of ganciclovir, reported a 43% reduction in neointimal formation. The induction of apoptosis by Fas ligand has also been exploited, as Sata *et al* (Sata, *et al.*, 1998) have very recently shown that the expression of Fas ligand will inhibit neointimal formation in rat carotid arteries following injury with a Fas ligand adenovirus impregnated balloon catheter. Pollman (Pollman, *et al.*, 1998), however, allowed vascular lesions to establish following catheter injury in the rabbit, prior to beginning therapy. This treatment, which involved the delivery of antisense bcl-x, induced intimal cell apoptosis and regression of both neointimal as well as primary atheromatous lesions.

While these reports are promising as novel therapeutic strategies, some issues remain to be addressed. For example, while most studies targeted the prevention of lesion formation, one
might speculate that in disease regression, as examined in Pollman's study, a marked physiological reduction in established vascular lesions would likely require both a decrease in cellularity and a loss of associated extracellular matrix. This was not addressed in that study, nor did the report establish whether a whole circulation, severely afflicted with disease, could be treated and improved. Such issues, however, are a focus of the research presented in this thesis and will be discussed in subsequent sections.
HYPOTHESES:

I. Along with the pro-migratory extracellular matrix glycoprotein, fibronectin, tenascin-C is upregulated in the pulmonary arteries of patients with pulmonary hypertension, related to congenital heart defects, and is co-distributed with growth factors and proliferating smooth muscle cells.

II. (a) Smooth muscle cells in their native environment, in pulmonary artery organ culture, will respond to physical changes by altering matrix metalloproteinase activity and tenascin-C production in concert with proliferation or apoptosis. Specifically, pulmonary arteries on attached collagen gels will induce matrix metalloproteinase activity and tenascin-C deposition, supporting smooth muscle cell proliferation. Conversely, in pulmonary arteries on floating collagen gels, matrix metalloproteinases will be suppressed, tenascin-C reduced and smooth muscle cell apoptosis induced.

(b) Hypertensive thick walled pulmonary arteries, with elevated serine elastase activity, may be more responsive to manipulation of tenascin-C in organ culture by attached versus floating conditions. Thus, elevated proteinase activity (specifically elastases and matrix metalloproteinases) and concomitant tenascin-C deposition on attached collagen will facilitate smooth muscle cell proliferation and continued vascular thickening, while floating conditions, with suppressed proteolysis, tenascin-C, and a profound induction of apoptosis will induce regression toward normal wall thickness (Fig. 0.8).

III. Inhibition of serine elastases or matrix metalloproteinases will suppress tenascin-C and induce smooth muscle cell apoptosis and loss of the accompanying extracellular matrix, thereby mediating regression of pulmonary artery hypertrophy. Suppression of tenascin-C directly will also induce apoptosis and regression of hypertrophy, as will αvβ3 integrin blockade.
IV. Suppression of smooth muscle cell apoptosis, induced by loss of ligation to tenascin-C following matrix metalloproteinase inhibition, is dependent on clustering of β3 integrins.

V. Regression of established pulmonary vascular disease can be achieved in the intact animal through administration of orally bioavailable, peptidyl trimethylfluoroketone serine elastase inhibitors.
**Figure 0.8**

*Hypothetical schematic illustrating the reversal of pulmonary vascular disease by the induction of smooth muscle cell apoptosis in response to suppression of tenascin-C either directly or indirect by serine elastase or matrix metalloproteinase inhibition.*
OBJECTIVES

I. To immunohistochemically characterize whether tenascin-C is upregulated during the progression of human pulmonary vascular disease and whether it is co-distributed with growth factors and proliferating vascular cells in the absence of evidence of apoptosis.

This will establish whether tenascin-C may be, as observed in cell culture and animal models, supporting vascular cell proliferation and thus a potential therapeutic target.

II. To determine whether medial thickness of either normotensive or hypertensive pulmonary arteries can be altered by regulating the proteinase-dependent deposition of tenascin-C using physical changes associated with the attached and floating collagen gel model.

III. (a) To use serine elastase and matrix metalloproteinase inhibitors to show that inhibition of these enzymes would be sufficient to induce regression of medial hypertrophy through the loss of tenascin-C and induction of smooth muscle cell apoptosis.

(b) To directly target tenascin-C, using an antisense ribozyme expression vector and HVJ-liposome transfection, and thereby establish the importance of this extracellular matrix ligand to smooth muscle cell survival.

(c) To determine if depriving smooth muscle cells of αβ3 ligation, with functional blocking antibodies, to survival factors like tenascin-C and osteopontin, will recapitulate the regression of medial hypertrophy observed with proteinase inhibitors.
IV. Using a previously established model of tenascin-C suppression and smooth muscle cell apoptosis, determine whether the induction of apoptosis is associated with unclustering of $\alpha v \beta 3$ integrins in a smooth muscle cell line and whether the artificial reclustering of these integrins will mediate survival of these cells despite matrix metalloproteinase inhibition.

V. To examine whether the delivery of serine elastase inhibitors can recapitulate organ culture results by suppression of smooth muscle cell survival ligands and induction of apoptosis and thus regression of vascular disease.

This will establish the therapeutic potential in using serine elastase inhibitors to treat clinical pulmonary hypertension.
CHAPTER ONE

Progressive Pulmonary Vascular Disease is Characterized by a Proliferative Response Related to Deposition of Tenascin-C and is Preceded by Subendothelial Accumulation of Fibronectin
ACKNOWLEDGEMENTS
The work presented in this chapter was performed under the supervision of a post-doctoral fellow, Dr. Peter L. Jones, whose guidance was of instrumental importance; he wrote this work in manuscript format while I began working out conditions by which to further explore these features in organ culture. I have adapted the text presented in this thesis, so that it is different from the published manuscript.

INTRODUCTION
Progressive pulmonary hypertension can develop as a result of various congenital heart defects in infants and children and remains a major complication in these patients despite early detection and surgical correction of the precipitating heart defect [reviewed in (Rabinovitch, 1995)]. The altered hemodynamics initiate a sequence of events that result in the development of structural abnormalities in the pulmonary vascular bed and initiate progressive increases in pulmonary vascular resistance leading to right ventricular hypertrophy and ultimately congestive heart failure. Underscoring the malignant progression of this disease are vascular smooth muscle cell (SMC) phenotypic changes. At the outset, muscularization of normally non-muscular peripheral arteries is mediated by the accumulation of SMCs through differentiation of precursor cells, pericytes and intermediate cells. In normally muscularized arteries, hypertrophy and hyperplasia of resident SMCs, together with heightened extracellular matrix (ECM) synthesis, culminates in increased medial wall thickness. Lesion development in the subendothelium resulting in occlusive vascular changes is related to migration of SMCs from the medial layer, recruitment of inflammatory cells, continued proliferation of SMCs as well as endothelial cells, and extensive ECM deposition.

While the underlying pathophysiology of pulmonary vascular disease is under intense investigation, numerous studies have suggested that its development may be related to
alterations in the composition of the vascular ECM (Todorovich-Hunter, et al., 1988; Prosser, et al., 1989; Maruyama, et al., 1991; Botney, et al., 1992; Tanaka, et al., 1996). A contribution from the ECM to the onset and progression of vascular lesions was suggested from studies like that of Botney et al (Botney, et al., 1992) examining lobar pulmonary arteries from patients with unexplained pulmonary hypertension obtained at the time of lung transplantation. The cellular neointimal lesions observed exhibited increased expression of tropoelastin, fibronectin (FN), thrombospondin, and type I collagen relative to the remainder of the vessel. Paralleled by extensive animal studies in rats and calves, increased deposition of elastin and collagen, following the onset of experimentally induced pulmonary hypertension, have been reported (Todorovich-Hunter, et al., 1988; Prosser, et al., 1989; Maruyama, et al., 1991; Tanaka, et al., 1996). This ECM deposition, which coincided with the development of structural vascular changes, was accompanied by heightened activity of serine class ECM-degrading proteinases (Todorovich-Hunter, et al., 1988; Maruyama, et al., 1991).

has been described in hypertensive vessels, these initial reports were restricted to the
documentation of alterations in TN synthesis (Hedin, et al., 1991), and to the study of
extrinsic factors which regulate its expression in tissue culture (Mackie, et al., 1992; Sharifi,
et al., 1992). Using rat pulmonary artery SMC primary cultures, our laboratory has
established that the addition of exogenous TN to type I collagen gels enhances the
proliferative response of SMCs to basic fibroblast growth factor (FGF-2), and is a
prerequisite for the mitogenic effects of epidermal growth factor (EGF) (Jones, et al.,
1997b). A role for TN in amplifying vascular SMC proliferation was further supported by
a progressive increase in TN mRNA and protein which co-localize with BrdU positive
SMCs during the initiation and progression of monocrotaline-induced pulmonary
hypertension in rats (Jones and Rabinovitch, 1996). The co-distribution of TN with
proliferating vascular SMCs has also been reported in venous anastomotic neointimal
hyperplastic lesions associated with polytetrafluoroethylene graft failure (Chen, et al.,
1996), as well as in spontaneously hypertensive rats, in which the co-localization of TN with
proliferating SMCs in the intact animal (Hahn, et al., 1995) was functionally associated with
a TN-mediated increase in mitogenic activity of epidermal growth factor and platelet-derived
growth factor in cell culture (End, et al., 1992). Thus, taken together, these reports suggest
that TN may provide a pro-proliferative microenvironment for vascular SMCs.

Alternatively, TN may be related to the induction of apoptosis. In studies of the mammary
gland during involution, the onset of apoptosis in mammary gland epithelial cells (Strange,
et al., 1992) is accompanied by the upregulation of TN expression which was responsible
for the loss of beta-casein protein synthesis, as reported by Jones et al (Jones, et al., 1995).
Consistent with this, TN induced apoptosis of fully differentiated mammary epithelial cells
in culture (Boudreau, et al., 1996). Given that restructuring of the vasculature at parturition
in lambs is associated with evidence of vascular cell apoptosis (Cho, et al., 1995) and
apoptosis is also observed in atherosclerotic plaques (Bennett, et al., 1995b; Geng and
Libby, 1995; Han, et al., 1995) and restenotic lesions (Bochaton-Piallat, et al., 1995a), where it may limit the proliferative response to injury, the possibility exists that TN counteracts cell proliferation in blood vessels by inducing apoptosis.

A role for FN-mediated SMC migration during neointimal formation has been established (Boudreau and Rabinovitch, 1991; Boudreau, et al., 1991; Clausell, et al., 1993a; Clausell, et al., 1994; Molossi, et al., 1995b; Hinek, et al., 1996; Cowan, et al., 1997). In intimal cushion formation in the fetal lamb ductus arteriosus, enhanced SMC transcriptional efficiency of FN (Zhou and Rabinovitch, 1998) during progressive cushion formation (Boudreau and Rabinovitch, 1991) is related to altered SMC shape and increased migration on type I collagen gels (Boudreau, et al., 1991). Moreover, during post-cardiac transplant coronary arteriopathy in piglets the development of intimal lesions is preceded by the establishment of a FN gradient between the vessel media and subendothelium (Clausell, et al., 1993a), suggesting that FN plays a critical role in the directional migration of SMCs into developing intimal lesions. Consequently, preventing the cytokine-mediated FN induction, through tumour necrosis factor-α blockade (Clausell, et al., 1994), or by blocking SMC and T-cell interaction with FN, by connecting segment-1 (FN) peptides (Molossi, et al., 1995b), attenuated post-transplant coronary artery disease.

Using a histological and immunohistochemical approach, we evaluated a potential role for TN and FN in the development and progression of human pulmonary hypertension. This was accomplished by examining a series of 7 lung biopsies derived for routine diagnostic purposes from children with various stages of pulmonary vascular disease, as judged by morphometric (Rabinovitch, et al., 1978) and Heath-Edwards (Heath and Edwards, 1958) criteria. Antibodies which recognize the proliferating cell nuclear antigen (PCNA), epidermal growth factor (EGF), (selected because of previous tissue culture studies), and apoptotic cells (by following nick end labeling) were used to relate the distribution pattern
and intensity of TN immunoreactivity to the presence of proliferating cells or those undergoing programmed cell death. A diffuse adventitial, focal periendothelial, or occasionally focal medial pattern of TN expression was observed in the thickened media, whereas PCNA and EGF positive cells were more widely distributed. In the neointimal however, the presence of intense TN immunoreactivity was co-distributed with foci of marked PCNA and EGF positivity. This finding supports a role for TN in neointimal formation as related to SMC proliferation. Fibronectin immunostaining increased with disease progression, being deposited in a periendothelial distribution. This observation is consistent with the presence of a FN gradient in promoting SMC migration from the media to the intima during the establishment of neointimal lesions. While only few T-cells and macrophages were documented, being restricted to the neointima of advanced occlusive lesions, apoptotic cells were absent, although the small sample size of tissue examined may be a consideration.
METHODS

Patients Studied and Histological Assessments

Lung biopsy tissue was obtained by cardiac surgeons at The Hospital For Sick Children from 7 patients (age range, 10 months to 13 years, 4 months; median age, 4 years 6 months) in which, on the basis of age and hemodynamic assessment, the severity of pulmonary vascular changes was of potential prognostic concern with regard to surgical correction. The samples were a series of consecutive biopsies, as available to us, and represented a range of vascular pathology. The protocol used in obtaining the lung biopsy tissue has been previously described (Rabinovitch, et al., 1978) and involved clamping the lung tissue in the inflated state in the right upper lobe to include a section of approximately 1 x 1 x 2 cm. The tissue was then rapidly fixed in the inflated state with warm 1% glutaraldehyde:4% formaldehyde for 10 minutes by a technique (Rabinovitch, et al., 1978) that enables assessment of the approximate vessel size when pressure fixation is technically impossible. These sections were then cut perpendicular to the clamp marks, further fixed in 10% neutral buffered formalin and subsequently embedded in paraffin, sectioned and stained by the Pathology Department, Hospital for Sick Children.

All lung biopsy sections were graded following Movat pentachrome staining using morphometric (Rabinovitch, et al., 1978) and Heath-Edwards (Heath and Edwards, 1958) criteria as follows: Morphometric Grade A: Extension of muscle into small peripheral arteries that are normally non-muscular, as previously quantified (Hislop and Reid, 1973; Rabinovitch, et al., 1978), or, in addition, there is a mild increase in wall thickness of the normally muscular arteries (less than 1.5 times normal); Grade B, as in grade A, there is increased extension of muscle, but, in addition, there is more severe medial hypertrophy of normally muscular arteries; Mild grade B: Medial thickness is greater than 1.5 but less than 2 times normal; Severe grade B: Medial thickness is more than twice normal; Grade C: In addition to the findings of severe grade B, arterial concentration and usually artery size are
reduced. Heath Edwards Grade I: Medial hypertrophy; Grade II: Cellular intimal proliferation; Grade III: Occlusive neointimal fibrosis; Grade IV: Dilatation complexes; Grade V: Angiomata formation.

**Immunohistochemistry**

For immunohistochemistry studies, 7μm sections from each biopsy were deparaffinized in xylene, sequentially rehydrated in graded alcohols (100-70%), PBS, pH 7.6, and PBS supplemented with 1% bovine serum albumin (BSA). Endogenous peroxidase was blocked by treating sections with 0.3% (v/v) hydrogen peroxide at room temperature for 30 min, followed by 2 washes in PBSA for 5 min each. Prior to detection of TN, FN, EGF, T cells, and macrophages, tissue sections were incubated at 37°C for 30 min with 1 mg/ml of pronase (Boehringer-Mannheim, Canada) in 0.05 M Tris base, pH 7.6, and then washed in PBSA for 10 min. For PCNA, tissue sections were incubated for 60 min at room temperature with nuclease solution (Amersham International plc, United Kingdom). To block non-specific binding, sections were incubated at 37°C for 30 min with PBSA containing 10% serum (Vector laboratories, Burlingame, CA). This step was omitted for detection of PCNA and α-smooth muscle actin.

Anti-human primary antisera which recognize tenascin-C (rabbit polyclonal serum, clone TN2, diluted 1/50; Gibco-BRL, Burlington, Canada), cellular fibronectin (rabbit polyclonal serum, dilution 1/50; Chemicon, Temecula, CA), proliferating cell nuclear antigen (mouse monoclonal IgG2a, clone PC10, diluted 1/100; Dako, Denmark), epidermal growth factor (mouse monoclonal IgG1, clone 144-8, diluted 1/50; Oncogene Science Inc., Uniondale, NY), α-smooth-muscle actin (horseradish peroxidase-conjugated mouse monoclonal IgG2, clone IA4, undiluted; Dako), T-cells (mouse monoclonal IgG2a, clone UCHL 1, diluted 1/50; Dako), and macrophages (mouse monoclonal IgM, clone HAM 56, diluted 1/50; Dako) were diluted in PBSA at the empirically determined optimal concentrations shown
above. Negative controls included omission of primary antibody and substitution of irrelevant antibody. All incubations were carried out overnight at 4°C. Thereafter, sections were washed in PBSA for 20 min and PBS for 10 min. Immunohistochemical detection of TN, FN, EGF, T-cells, and macrophages was carried out using species-specific reagents based on the avidin-biotin peroxidase method (Vector Laboratories). For PCNA staining, samples were incubated with a peroxidase conjugated anti-mouse IgG prepared according to the manufactures' instructions (Amersham International Plc.). All immunostained sections were then exposed to 0.5 mg/ml of diaminobenzadine diluted in 0.05 M tris buffer, pH 7.6, containing 0.02% hydrogen peroxide and lightly counterstained with haemotoxylin or eosin for PCNA.

All preacinar arteries and those accompanying terminal and respiratory bronchioli in each section (usually between 15 -20) were analyzed. TN and PCNA immunostaining were examined in serial tissue sections within the same vessel, whereas EGF staining was examined in sections from the same biopsies which contained similar vessels. Immunostaining for TN, FN, EGF, and PCNA was graded as negative (-), modest (+), moderate (++), intense (+++), very intense (++++) and the distribution of TN, FN and EGF was also recorded as diffuse or focal and localized to adventitia, media, or intima. An average grade and distribution was then ascribed for each section, by a consensus of three independent blinded observers (PLJ, KNC, MR). However, the nature of the lesions made it difficult to be blinded to the grade of biopsy. The presence of T cells or macrophages was noted within the vessel wall and localized to the intima, media, or adventitia. Alpha-smooth muscle actin was also used to determine whether the actively proliferating cells or the cells that stained intensely with marker for TN, FN, or EGF were likely to be smooth muscle or non-smooth muscle in origin.
Detection of Apoptotic Cells

DNA fragmentation is a characteristic feature of apoptotic cells and was assessed using the TUNEL assay (Apoptag kit, Oncor Inc., Gaithersburg, MD). Briefly, formalin-fixed tissue sections were deparaffinized in xylene, washed in graded ethanols (100-70%), and PBS, pH 7.6. Autofluorescence was quenched by incubating sections in 0.1 M glycine in PBS, pH 7.6. Sections were treated with 20 mg/ml of proteinase K for 15 min at room temperature, washed in distilled water and equilibrated with equilibration buffer for 5 min at room temperature. Next, sections were incubated in equilibration buffer for 1 h at 37°C with terminal deoxy-nucleotidyltransferase (TdT) in the presence of digoxigenin-11-dUTP and dATP, and thereafter in stop buffer for 30 min at 37°C. Sections were incubated with a fluorescein-conjugated anti-digoxigenin antibody for 30 min at ambient temperature. Formalin-fixed, paraffin-embedded, full thickness human skin sections were used as a positive control. Substitution of distilled water in place of TdT enzyme served as a negative control. To detect all nuclei, sections were incubated for 20 min in 4',6-diamidino-2-phenylindole (DAPI) diluted 1/10 000 in PBS, and were then washed in PBS for 30 min being viewed by epifluorescence using standard fluorescein excitation and emission filters.
RESULTS

Patients studied and histological assessment of graded vascular lesions

Lung biopsies were examined from two patients who had a secundum atrial septal defect (ASD), three with complete atrioventricular septal defects (AVSD), one with transposition of the great arteries (TGA) together with a ventricular septal defect (VSD), and one having a univentricular heart. The 3 AVSD patients had lung biopsy grades of I (Heath Edward), C (morphometric), IIIIC and IVC. The 2 ASD patients' biopsies were graded 0-IA and IB, the TGA graded IV-VC and the single ventricle was designated IIB. Mean pulmonary artery pressures ranged from 15 to 71 mmHg and typical histological findings are shown in Figure 1.1, with specific clinical and hemodynamic data provided in Table 1.1.

Localization of tenascin-C, proliferating cell nuclear antigen, epidermal growth factor and cellular fibronectin

In low grade lesions, immunohistochemistry for TN indicated that TN first appeared in the adventitia (Grade 0-IA) (Figure 1.2A), and progressed into both the adventitia and media where its deposition become more focal in nature (Grade IB). Further disease progression, reflected as an increase in lesion grade, was related to a more intense TN immunoreactivity with a periendothelial distribution (Grade IC) (Figure 1.2D) that increased in intensity with the development of a neointima (Grades II-IV) (Figure 1.2G). Proliferating cell nuclear antigen positivity was, however, absent in the lowest grade lesions (IA) (Figure 1.2B), despite moderate staining for EGF in the media (Figure 1.2C). Proliferating cells became evident in the media, or as foci in the periendothelial region and adventitia (Figure 1.2E). This PCNA immunoreactivity did not necessarily co-localize with TN or EGF in these early lesions (Grade IB, IC, II B) (Figure 1.2F). With the development of occlusive changes, represented by higher grades (Grade III-V), PCNA (Figure 1.2H) and EGF (Figure 1.2I) expression were most intense in the neointima where they co-localized with TN (Summarized in Table 1.2). Recent evidence suggests that fixation method may alter the
degree of PCNA staining, specifically, the method used in this study may have preserved the cells such that any cycling cell would be detected as PCNA positive, not just those in S phase. This feature is an important consideration in the interpretation of data and may provide further evidence of heterogeneity in vascular SMC subpopulations, given that a population of SMCs was therefore selectively induced to enter the cell cycle, constituting the cells of the neointimal lesion, while other resident SMCs remained quiescent.

Immunostaining for FN was present in all biopsy samples regardless of the severity of lesion grade. This positivity, which was primarily restricted to the immediate periendothelial region (Figure 1.3A-D), was modest in early lesions, grade 0-IA (Figure 1.3A), and moderate to intense in all lesions exceeding grade IB (Figure 1.3B & C). With the establishment of neointimal occlusive lesions, grades II-IV, FN, while intensely deposited and remaining localized within the periendothelial space, was also diffusely distributed throughout the neointima (Figure 1.3D) (Summarized in Table 1.2).

**Cellular composition of vascular lesions**

The cellular composition of vascular lesions was characterized by immunohistochemical studies with antibodies against α-smooth muscle actin, T-cells, and macrophages. While α-smooth muscle actin positive cells consistently predominated within the media and neointima of all vessels examined (Figure 1.4A & B), evidence of T-cells and macrophages within pulmonary arteries was restricted to an occasional positive cell in the neointima of higher grade lesions (Figure 1.5A & B). In low grade lesions, any immunoreactivity for T-cells and macrophages was only present in the periadventitia.

**Apoptosis may not be a prominent feature of human pulmonary vascular disease**

In recent studies, a role for SMC apoptosis in vascular remodeling has been suggested by the presence of apoptotic cells during vascular development, in response to altered
hemodynamics (Cho, et al., 1995), and vascular pathologies (Bennett, et al., 1995b; Bochaton-Piallat, et al., 1995a; Geng and Libby, 1995; Han, et al., 1995). In the present study, using TUNEL assays to detect apoptotic cells in lung biopsy tissue, no apoptosis was observed in any of the samples examined irrespective of lesion grade (Figure 1.6A). Absence of apoptosis was confirmed by lack of condensed or fragmented 'apoptotic' nuclei by propidium iodide labeling, an alternative method for assessing apoptosis (data not shown), and by the presence of TUNEL positive apoptotic nuclei in normal full thickness human skin sections (Figure 1.6B), which were prepared in an identical manner to rule out the possibility that our negative finding was related to tissue preservation.
Figure 1.1

Representative photomicrographs of lung biopsy tissue following Movat pentachrome staining. Vessels were graded according to Heath-Edwards and morphometric criteria (see Materials and Methods). (A) Vessel showing a typical Grade IA lesion with minimal medial hypertrophy. (B) Vessel showing a typical Grade IB lesion showing severe medial hypertrophy. (C) Vessel showing a typical Grade III C lesion with extensive neointimal formation. (D) Vessel from a typical Grade IV C lesion with occlusive neointimal fibrosis and medial atrophy (part of a plexiform complex). Original magnification = x40.
Table 1.1

Clinical, hemodynamic, and lung biopsy findings.
### Table 1  Clinical, Hemodynamic and Lung Biopsy Findings

<table>
<thead>
<tr>
<th>Age (y/m)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Hemodynamic Data, Lung Biopsy Grade</th>
</tr>
</thead>
</table>
| 2y 2m    | M   | ASD chronic lower respiratory tract infections | Ppa - 40mmHg  
Qp: Qs = 2:8:1  
Heath Edwards 0-1  
Morphometric A |
| 13y 4m   | F   | ASD cardiomyopathy                | Ppa - 51mmHg  
Qp:Qs = 3:1  
Heath Edwards I  
Morphometric B |
| 2y 7m    | F   | AVSD                             | Ppa - 71mmHg reactive to O2  
Qp:Qs = 6:1  
Heath Edwards I  
Morphometric C |
| 1y 4m    | M   | Single LV Absent (L) AV valve S/P PA band | Ppa - 15mmHg distal to PA band  
Heath Edwards II  
Morphometric B |
| 10 m     | M   | AVSD                             | no catheter study  
Heath Edwards III  
Morphometric C |
| 3y 3m    | F   | AVSD                             | Ppa - 48mmHg distal to PA band  
Qp:Qs = 3:2:1  
Heath Edwards IV  
Morphometric C |
| 8y 1m    | M   | TGA+VSD S/P PA band              | Ppa - 45mmHg distal to PA band  
Qp:Qs = 8:1  
Heath Edwards IV-V  
Morphometric C |

Abbreviations: y=year, m=month, ASD=atrial septal defect, AVSD=atrioventricular septal defect, RV=right ventricle, S/P=statis post, PA=pulmonary artery, Ppa=mean pulmonary artery pressure, Qp:Qs=pulmonary to systemic flow ratio, TGA=transposition of the great arteries. Grading of biopsies is described in Materials and Methods
Figure 1.2

Representative photomicrographs showing immunoperoxidase staining for tenascin-C (TN) (A, D and G), proliferating cell nuclear antigen (PCNA) (B, E and H) and epidermal growth factor (EGF) (C, F and I) in graded lung biopsy tissue sections. A, B and C: Vessel showing a typical Grade IA lesion; D, E and F: Vessel showing a typical Grade IC lesion; G, H and I: Vessel showing a typical Grade III C lesion. In low grade lesions (A), modest TN immunostaining was evident in the adventitia. With medial hypertrophy, TN immunoreactivity became more prominent in the periendothelium (D), with the most intense immunostaining being apparent within the neointima of high grade lesions showing occlusive neointimal formation (G). In the lowest grade of lesion, PCNA was negative (B), despite foci of EGF in the media. With medial hypertrophy, PCNA was expressed in the media (E), together with foci of EGF (F). With the development of higher grade occlusive lesions, TN (G), PCNA (H) and EGF (I) co-localized to the neointimal cell layers. Note that TN and PCNA staining was performed on serial sections, whereas EGF detection was carried out on similar vessels within the same biopsy. Original magnification = x40.
Table 1.2

*Expression and localization of tenascin-C, proliferating cell nuclear antigen, epidermal growth factor, and cellular fibronectin.*
Table 2 Expression and localization of tenascin-C, proliferating cell nuclear antigen, epidermal growth factor and cellular fibronectin

<table>
<thead>
<tr>
<th>Biopsy grade</th>
<th>Tenascin</th>
<th>PCNA</th>
<th>EGF</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-I A</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>adventitia</td>
<td></td>
<td>focal in media</td>
<td>periendothelium</td>
</tr>
<tr>
<td>1 B</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>focal in adventitia &amp; media</td>
<td>media</td>
<td>diffuse in media, focal in peri-endothelium &amp; adventitia</td>
<td></td>
</tr>
<tr>
<td>1 C</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>periendothelium &amp; adventitia</td>
<td>periendothelium</td>
<td>focal in media &amp; adventitia</td>
<td></td>
</tr>
<tr>
<td>11 B</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>adventitia &amp; neointima</td>
<td>media &amp; adventitia</td>
<td>focal in media periendothelium</td>
<td></td>
</tr>
<tr>
<td>111C</td>
<td>++++</td>
<td>++++/++++</td>
<td>+++</td>
<td>++++/++++</td>
</tr>
<tr>
<td></td>
<td>neointima</td>
<td>media &amp; neointima</td>
<td>focal in neointima</td>
<td>periendothelium &amp; neointima</td>
</tr>
<tr>
<td>11VC</td>
<td>++++/+++</td>
<td>+++</td>
<td>++++/+++</td>
<td>++++/+++</td>
</tr>
<tr>
<td></td>
<td>adventitia &amp; neointima</td>
<td>media &amp; neointima</td>
<td>focal in adventitia &amp; neointima</td>
<td>periendothelium &amp; neointima</td>
</tr>
<tr>
<td>111-V C</td>
<td>++++/+++</td>
<td>++++/+++</td>
<td>++++/+++</td>
<td>++++/+++</td>
</tr>
<tr>
<td></td>
<td>media &amp; neointima</td>
<td>media &amp; neointima</td>
<td>media &amp; neointima</td>
<td>periendothelium &amp; neointima</td>
</tr>
</tbody>
</table>

Key: Immunostaining for tenascin-C (TN), cellular fibronectin (FN), epidermal growth factor (EGF), and proliferating cell nuclear antigen (PCNA) was graded as negative (-), modest (+), moderate (++), intense (+++), or very intense (++++). Biopsy grade is described in Materials and Methods.
**Figure 1.3**

*Representative photomicrographs showing immunoperoxidase staining for cellular fibronectin (FN) in graded lung biopsy tissue sections.*  
A: Vessel showing a typical Grade IA lesion; B: Vessel showing a typical Grade IC lesion; C: Vessel showing a typical Grade IIIIC lesion; D: Vessel showing a typical Grade IVC lesion. Modest to intense periendothelial FN immunostaining was apparent in grade I lesions (A and B). In higher grade lesions (C and D), more intense FN immunostaining was observed in the periendothelial and neointimal cell layers. Original magnification = x40.
Figure 1.4

Representative photomicrographs showing immunoperoxidase staining for α-smooth muscle actin in graded lung biopsy tissue sections. A: Vessels showing typical Grade I B lesions; B: Vessel showing a typical Grade IV C lesion. Medial smooth muscle cells (A) and neointimal lesions (B) were comprised predominantly of cells that stained for α-smooth muscle actin. Original magnification = x40.
Figure 1.5

*Representative photomicrographs showing immunoperoxidase staining for T-cells and macrophages in a typical Grade IVC lesion showing obstructive neointimal formation.* Low level infiltration of T-cells (A) and macrophages (B) was observed in the adventitial/outer medial and neointimal cell layers. Arrows indicate the presence of inflammatory cells. Original magnification = x40.
Figure 1.6

*Representative photomicrographs showing Apoptag (green) and DAPI (blue) staining in a vessel showing a Grade I\textsuperscript{B} lesion (A), and in full thickness human skin (B).* No apoptotic nuclei, (identified by green and blue double staining), were present in remodeling blood vessels (A), but were evident in full thickness skin (B). Arrows indicate presence of apoptotic cells. *Original magnification = x40.*
DISCUSSION

In the present study, the development and progression of pulmonary vascular disease, secondary to congenital heart defects, in children was shown to be associated with increased expression of TN, PCNA and EGF. In lower grade biopsies, the onset of medial hypertrophy was, however, inconsistently associated with the co-distribution of these markers. This suggests that with such early abnormalities additional factors, aside from TN, likely contribute to their establishment. Nevertheless, with neointimal lesions, a critical pro-proliferative role for TN is supported by the intense co-distribution of TN with both EGF and PCNA. The consistent periendothelial distribution and accumulation of FN that precedes the advent of neointimal formation suggests the formation of a gradient which favours SMC migration into the expanding lesion. Using α-smooth muscle actin as a marker, we have established that these highly cellular lesions are predominantly smooth muscle in origin. Only in high grade lesions do neointimal T-cells and macrophages become evident. In addition, our inability to detect apoptotic cells in these lesions suggests that apoptosis is not a readily identifiable feature in the progression of pulmonary hypertension as a result of congenital heart defects.

In this study, we have examined biopsies from seven children exhibiting pulmonary vascular abnormalities. While this may represent a limitation to the study, the changes observed within each sample were, however, consistently evident in a large number of vessels examined in each tissue and, together, represent the full spectrum of lesion grades and disease. Moreover, additional studies from our laboratory and others documenting intimal changes associated with vascular lesion development and progression support regional heterogeneity in the distribution of various ECM proteins (Boudreau, et al., 1991; Botney, et al., 1993; Clausell, et al., 1993a; Liptay, et al., 1993; Clausell, et al., 1994; Durmowicz, et al., 1994; Riesen, et al., 1994; Molossi, et al., 1995b; Cowan, et al., 1997). Specifically, in the fetal lamb ductus arteriosus at gestation (Boudreau and Rabinovitch, 1991), and in
coronary arteries of piglets (Clausell, et al., 1993a; Clausell, et al., 1994) and rabbits (Cowan, et al., 1997) following heterotopic cardiac transplantation. FN production increased and accumulated in the periendothelium preceding intimal formation. Consistent with these observations, we now report for the first time in clinical tissue, by including low grade vascular lesions in our analyses, that accumulation of FN in the periendothelium is an early feature of pulmonary vascular disease which may favour SMC migration into the developing neointima.

We have recently shown that TN initially co-localizes with proliferating SMCs in the adventitial and outer medial cell layers in the pulmonary arteries of adult rats during the development of monocrotaline-induced pulmonary hypertension (Jones and Rabinovitch, 1996). Moreover, these features extend to the intima with progressive medial hypertrophy. The expression of TN may be indirectly related to heightened serine elastase activity, previously described in association with experimentally-induced vascular lesions (Oho and Rabinovitch, 1994; Zhu, et al., 1994; Oho, et al., 1995). Tissue culture studies have shown that serine elastases liberate basic fibroblast growth factor (bFGF) from ECM stores (Thompson and Rabinovitch, 1996) and bFGF is a potent SMC mitogen which is also known to stimulate TN synthesis (Meiners, et al., 1993; Tucker, et al., 1993). In this study, we have focused on EGF, another SMC mitogen (Scott-Burden, et al., 1988), since it is expressed in vascular lesions in regions of cell proliferation and our recent tissue culture studies indicate that TN is required for EGF-responsiveness of rat pulmonary artery SMCs (Jones and Rabinovitch, 1996). Thus, rather than a marker of cell proliferation in response to EGF, we would suggest that TN is an active participant. Based upon alpha smooth muscle actin immunostaining, both in these studies and in the previous monocrotaline experiments, where in situ hybridization was carried out, the major source of TN appears to be the vascular smooth muscle cells. Mechanistically, the effect of TN on EGF-dependent SMC growth is mediated in part by the αvβ3 integrin receptor, and is accompanied by
reorganization of the filamentous actin cytoskeleton, and increased EGF receptor clustering, a prerequisite for efficient EGF mitogenic signaling. Together, these data suggest that the TN-dependent effects physically and biochemically prime SMCs so that EGF receptors rapidly phosphorylate following addition of their cognate ligand (Jones, et al., 1997b).

The infiltration of T-cells and macrophages is a common feature of neointimal lesions in coronary arteries following cardiac transplant (Clausell, et al., 1994; Molossi, et al., 1995b; Cowan, et al., 1997) as well as in human pulmonary arteries with advanced disease, analyzed from tissue received at transplant (Liptay, et al., 1993). Given that their presence co-localizes with ECM neosynthesis, recruitment of inflammatory cells may be related to ECM accumulation. However, in the human pulmonary hypertensive lesions examined in the present study, only the occasional T-cell or macrophage was observed in advanced grade lesions, being restricted to the outer medial and adventitial cell layers. Moreover, these cells are entirely absent from lower grade lesions. As TN and FN expression is upregulated by interleukin-1β in various cell types (Rettig, et al., 1994), including pulmonary artery SMCs (unpublished observations from our laboratory), cytokine-mediated ECM deposition may therefore contribute to the ECM remodeling observed (Clausell, et al., 1993a; Lonnemann, et al., 1995).

Given the considerable heterogeneity of vascular SMCs reported (Glukhova, et al.,; Betz, et al., 1991; Frid, et al., 1994; Perronneau, et al., 1995; Wohrley, et al., 1995), focal patterns of TN and EGF localization may represent the preferential response of unique subpopulations. As SMCs respond similarly (Jones, et al., 1997b) to chick embryo fibroblasts which have been reported by Chiquet-Ehrismann et al (Chiquet-Ehrismann, et al., 1994) to downregulate TN protein synthesis when cultured on stress-relaxed type I collagen gels, mechanical factors may regulate ECM deposition within the vessel wall. Consistent with this, increased TN and FN expression in the present study correlated with
elevated mechanical stress and hemodynamic changes that accompany progressive pulmonary hypertension. Moreover, removal of mechanical stress, following correction of the precipitating cardiac defect, may result in regression of vascular lesions through suppression of TN.

Although it is widely believed that SMC apoptosis in blood vessels contributes to tissue homeostasis (Schwartz and Bennett, 1995) and regression of vascular lesions, the present work, as well as our studies with the development of pulmonary vascular disease in monocrotaline-treated rats (Jones and Rabinovitch, 1996), indicates that SMC apoptosis may not be a prominent feature of pulmonary hypertension. Recent work has shown that cell ligation to FN, via the α5β1 integrin receptor promotes cell survival and suppresses apoptosis by bcl-2 upregulation (Zhang, et al., 1995), and endothelial cell ligation through a TN receptor, the αvβ3 integrin (Sriramarao, et al., 1993), inhibits the apoptotic machinery as a consequence of downregulation of p53 and an increased bcl-2 to bax ratio (Brooks, et al., 1994b). Given such evidence, the presence of both TN and FN during neointimal lesion formation may serve to support vascular SMC survival accounting for our inability to detect apoptotic SMCs. Taken together, these studies suggest that TN, FN, and their cognate receptors may represent novel therapeutic targets for preventing the development of vascular disease. The process by which SMCs perceive and integrate signals derived from FN and TN are fundamental questions currently being investigated.
CHAPTER TWO

Regression of Hypertrophied Rat Pulmonary Arteries in Organ Culture is Associated with Suppression of Proteolytic Activity, Inhibition of Tenascin-C and Smooth Muscle Cell Apoptosis
ACKNOWLEDGEMENTS

For the work presented in this chapter, I would like to acknowledge Dr. Peter L. Jones's helpful discussions and critical review of samples.

INTRODUCTION

Pulmonary hypertension (PH) resulting from diverse etiologies can be a rapidly progressive and fatal disease (reviewed in reference (Rabinovitch, et al., 1986)). It is characterized by changes in vascular function and structure associated with endothelial cell injury, (Rabinovitch, 1997) neomuscularization of normally non-muscular peripheral arteries, and smooth muscle cell (SMC) proliferation, hypertrophy and migration into the neointima (reviewed in reference (Rabinovitch, et al., 1986)). This is accompanied by remodeling of the extracellular matrix (ECM) by proteinases (Rabinovitch, 1997), most notably endogenous serine elastases (Zhu, et al., 1994; Thompson, et al., 1998), and by increased expression and deposition of ECM components, elastin, collagen (Todorovich-Hunter, et al., 1988; Prosser, et al., 1989), fibronectin (Botney, et al., 1992; Jones, et al., 1997a), and tenascin-C (TN) (Jones and Rabinovitch, 1996; Jones, et al., 1997a).

Our laboratory has studied the pathophysiologic role of tenascin-C (TN) in pulmonary vascular disease (PVD) and its relationship to cellular apoptosis and proliferation. In lung biopsies from patients with pulmonary hypertension, expression of TN was observed in the hypertrophied media and neointima in association with proliferating cell nuclear antigen (PCNA) positive cells (Jones, et al., 1997a). Similar observations were made in experimentally-induced progressive pulmonary hypertension in rats following monocrotaline (MCT) injection (Jones and Rabinovitch, 1996). A cause and effect relationship between TN and SMC proliferation was shown in cell culture studies, where the incorporation of TN in the ECM amplified the proliferative response to growth factors (Jones and Rabinovitch, 1996). The mechanism was related to an alteration in SMC shape
and was further explored by comparing cells on attached or floating type I collagen gels (Jones, et al., 1997b). In intact collagen gels, MMP-2 activity and ligation of \( \beta_3 \) integrins by cryptic RGD sites in proteolyzed collagen leads to TN production. Clustering of \( \alpha_\gamma \beta_3 \) integrins, by newly synthesized TN, was associated with focal adhesion contact formation. Growth factor (EGF) receptors are consequently also clustered and efficient receptor phosphorylation occurs following ligation. Subsequent tyrosine phosphorylation and nuclear growth signals are evident. Alternatively, on floating collagen MMP-2 activity declines, endogenous TN levels fall and SMC apoptosis is evident (Jones, et al., 1997b).

These studies raise the question as to whether SMCs in their native vascular environment might also respond to physical changes by altering MMP and TN production in concert with proliferation or apoptosis. To investigate this, we cultured intact main pulmonary arteries (PAs) on attached or floating collagen gels and assessed MMP activity as well as TN expression and SMC proliferation. In PAs on attached collagen, we documented increasing MMP-2 and -9 activity, by gelatin substrate zymography and reverse zymography, and deposition of TN, by western immunoblot and immunohistochemistry. In contrast, in floating cultures, suppression of TN and a relative reduction in activity of both MMPs was observed. The presence of both TN rich and TN poor foci correlated with proliferation and apoptosis respectively, but these effects did not appear to significantly impact on medial wall thickness in normal vessels.

We reasoned, however, that hypertensive vessels, already in a state of active remodeling, may be more responsive to manipulation of TN in organ culture by attached versus floating conditions. In addition, an amplified deposition of TN may occur through serine elastase activity, which is increased in hypertensive PAs (Zhu, et al., 1994; Thompson, et al., 1998) and may induce heightened activation of MMPs (Okada, et al., 1988; Okada and Nakanishi, 1989) or might directly upregulate TN by the mechanism proposed in cell culture studies.
This increased deposition of TN may, in turn, amplify the SMC proliferative response. Conversely, withdrawal of TN could mediate a more profound apoptotic response if cells from diseased vessels behave similarly to cells in organ culture.

To investigate this, we cultured hypertrophied rat main PAs 21 days following injection of the toxin MCT, on attached or floating collagen gels and collected the tissue 3, 5, and 8 days later for analysis. The progressive medial thickening observed in rat PAs following MCT injection continued on attached collagen gels, while vessels placed on floated gels underwent a regression of medial thickness to control (saline) levels. This was associated with a suppression of elastase and MMP activity (primarily MMP-2), and loss of TN assessed by northern blot, western immunoblot and immunohistochemistry. Regression of hypertrophy was furthermore associated with reduced elastin indicating that a coordinated loss of both vascular cellularity and extracellular matrix components was achieved.
MATERIALS AND METHODS

Preparation of Explants:
Pulmonary arteries were collected and prepared for organ culture as previously described (Gotlieb and Boden, 1984). Freshly harvested porcine main PAs obtained from the local slaughterhouse were transported to the laboratory in sterile phosphate-buffered saline (PBS) and 2% antibiotics/antimycotics (Gibco Laboratories, Grand Island, NY). The explants were prepared under sterile conditions, keeping the endothelial surface moist at all times and taking care not to damage this surface. The periadventitial fat and adventitia were removed gently from unopened PAs. The PAs were then cut along their long axes and pinned out, endothelial side uppermost, taking care not to stretch the vessel. Rectangular pieces of full thickness vessel wall (4 x 10 mm) were then cut using a scalpel blade from a site away from the ends of the vessel, and these sections were washed thoroughly in PBS, and placed in culture medium.

In the second series of experiments, main pulmonary arteries were harvested from adult male Sprague-Dawley rats (300 g) (Charles River Laboratories, St. Laurent, CN) 21 days following a subcutaneous injection of the alkaloid toxin monocrotaline (60 mg/kg) (MCT) or saline (controls). Monocrotaline-induced PVD is well established (Meyrick, et al., 1980; Kido, 1981) and at 21 days after injection of the toxin a doubling of the wall thickness of the pulmonary artery is expected (Todorovich-Hunter, et al., 1988). The rats were sacrificed by a lethal intraperitoneal injection of sodium pentobarbital and central pulmonary arteries were harvested, cleaned, and placed in cold, sterile PBS and 2% antibiotics/antimycotics (Gibco), then handled in the same manner described above for the porcine vessels. This procedure was in accordance with the guidelines of the Animal Care Committee of the Hospital for Sick Children, Toronto, Canada.
Each explant was cultured in a 35 mm dish while embedded in a type I collagen gel (Vitrogen 100, Collagen Corp., Fremont, CA). The collagen gel was prepared according to the manufacturer's recommendation. Briefly, vitrogen 100 collagen was mixed on ice with 10X PBS, containing 0.005 mg/ml phenol red, and 0.1 mol/L NaCl at a ratio of 8:1:1 and adjusted to pH 7.4. The neutralized collagen solution was then added to ice-cooled culture dishes (2 ml aliquots for porcine PAs and 1 ml for rat PAs) and dispersed using a cell spreader. Tissue slices were placed endothelium side up in the viscous solution and subsequently settled, sinking slightly into the gel during collagen fibrillogenesis, which was initiated by warming in a cell culture incubator at 37°C for two hours. Consequently, once fibrillogenesis was complete, the tissue slices were located at the interface between the atmosphere and the top of the gel and 2.5 mls of Medium 199 (Gibco) were carefully added to each dish, avoiding disruption of the collagen gel. The media was replaced after one hour, supplemented with 5% fetal bovine serum, 2% antibiotics/antimycotics and additional 0.2% gentomycin (Gibco) and changed every three days. After one day, half the cultures were 'floated'. The medium was removed and the edges of the gel were released by moving the end of a sterilized spatula around the perimeter of the dish. Then, by gradually teasing the edges of the gel with the spatula the bottom of the gel was separated from the culture dish and medium was reapplied. Pulmonary arteries and conditioned media from attached and floating cultures were collected at 3, 5, and 8 days.

**Movat Pentachrome Staining**

Tissue samples were stained by the Movat pentachrome method and examined by light microscopy. The Movat stain includes iron haematoxylin and thus identifies the location of both the internal and external elastic laminae as well as the medial elastin fibers. Measurements of thickness of both intima (from upper surface of endothelium to top of internal elastic lamina) and media (from upper margin of internal elastic lamina to top of external elastic lamina) were calculated. In porcine pulmonary arteries, 3 equidistant
measurements over the length of the vessel were recorded for each tissue and in rat pulmonary arteries, 25 measurements in consecutive fields of view (290 μm² fields at an original magnification of 400x) were recorded and means calculated. Elastic lamellae were assessed by counting the number of continuous elastic laminae per pulmonary artery. That is, in cases where elastic laminae separated and fused, they were counted as one. The total amount of elastin was assessed by planimeterization of both elastic laminae and interlamellar fragments of elastin. These assessments were performed in 5 random fields per vessel at the same magnification indicated above.

Assessment of Elastolytic Activity
Elastase assays were performed using 380 μl of conditioned media from attached and floating cultures and incubated at 37°C with 20 μl (200 μg) [³H]-elastin produced by radiolabeling purified insoluble elastin from bovine nuchal ligament (Elastin Products Co., Owensville, MO) using [³H]-NaBH₄ (New England Nuclear, Boston, MA) as previously described (Takahashi, et al., 1973). After 24 hours, 250 μl of the supernatant was collected following microcentrifugation at 8,160 g for 5 minutes. The radioactivity of the supernatant was determined by liquid scintillation spectrometry. To control for non-enzymatic degradation of the elastin substrate, assays were also carried out incubating radiolabeled elastin with fresh medium alone. Samples were performed in triplicate. Activity was related to a standard curve generated with human leukocyte elastase (0.075-5.0 ng) (Elastin Products Co.).

Matrix Metalloproteinase Gelatin Substrate Zymography
Tissue extracts were made by homogenizing PAs at 4°C in homogenization buffer (50 mmol/L Tris-Cl, 0.2% Triton X-100, 10 mmol/L CaCl₂, 2 mol/L Guanidine HCl, pH 7.5). Samples were then lyophilized overnight, normalized for protein content by Bradford assay, and separated on a non-reducing, non-denaturing 10% polyacrylamide gel containing 0.1%
gelatin (Sigma). Following electrophoresis, gels were washed in 2.5% Triton X-100 and incubated in substrate buffer (50 mmol/L Tris-Cl, pH 7.5, 5 mmol/L CaCl₂) for 48 hours at 37°C. Clear gelatinolytic bands were identified by staining gels with Coomassie Blue R250 (Biorad). To identify tissue inhibitors of MMPs (TIMPs), gels were prepared and proteins electrophoresed as described above, but then incubated in (4-aminophenyl) mercuric acetate (APMA) activated conditioned medium for 3 hours at 37°C. Dark anti-gelatinolytic bands were identified by staining gels with Coomassie Blue R250 (Biorad).

**Western Immunoblotting**

Identification of MMPs 2 and 9 and TIMP-1 by western immunoblot was performed by resolving proteins from tissue extracts on a 12% polyacrylamide gel followed by transfer to an Immobilon-P membrane (Millipore). Immunoblotting was performed using anti-human MMP-2 and -9 and TIMP-1 monoclonal antibodies (1:50) (Oncogene Science, Uniondale, NY) for porcine extracts and anti-MMP-2 rabbit polyclonal antisera for rat extracts (a kind gift from Drs. M. Silverman and M. Ailenburg, Department of Medicine, University of Toronto, Toronto, Canada). Immunoblotting was also performed with an anti-human cellular TN polyclonal antibody (1:50) (Sigma Chemical Co., St. Louis, MO) for porcine tissue and an anti-rat polyclonal antibody raised against formaldehyde-fixed TN (1:100) (a generous gift from Dr. Harold Erickson, Duke University Medical Center, Durham, NC) after blocking the membrane for 1 hour at room temperature in wash buffer (10 mmol/L Tris, pH 7.5, 10 mmol/L NaCl, and 0.1% Tween-20) containing 5% milk. Following a 1 hour room temperature incubation with the primary antibody, binding was visualized by ligation with a horseradish peroxidase-conjugated secondary antibody (Gibco-BRL, Burlington, ON) at 1:2500 dilution in wash buffer. Visualization of immunoreactive protein bands was achieved by enhanced chemiluminescence (Amersham Corp.) and normalization for protein content was performed by comparison with an identically loaded, non-transferred Coomassie Blue stained gel.
**Northern Blotting**

Total RNA for northern blot analysis was extracted from PAs cultured under attached and floating conditions. PAs were first washed twice in PBS and immediately placed in 5 mL of TRIzol (Gibco-BRL). Samples were homogenized with a polytron homogenizer and mixed with 1 mL of chloroform and centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous phase was mixed vigorously with 2.5 mL of isopropanol and allowed to stand at room temperature for 10 minutes. The samples were then centrifuged at 12,000 g for 15 minutes at 4°C, and the resulting RNA pellets were washed in 75% ethanol. An 8 µg sample of total RNA per lane was loaded on a 0.8% agarose/formaldehyde gel and transferred to a nylon membrane (Hybond-N+, Amersham International) by capillary transfer for 12 hours and then cross-linked by exposure to UV radiation. Hybridizations were performed with a [32P]-labeled random-primed probe prepared from a 250-bp cDNA derived from the seventh fibronectin type III constant domain of rat TN. The relative quantity of TN mRNA in each sample was analyzed by densitometry and corrected for loading conditions by direct comparison with measurements of 28s rRNA, detected after ethidium bromide staining of agarose gels, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), following hybridization with a 600bp cDNA probe for rat GAPDH.

**Immunohistochemistry**

All samples were removed from the collagen gels and fixed in 2% paraformaldehyde. They were then embedded in paraffin and subsequently cut into 5µm thick sections. Immunohistochemistry was also performed with the TN antibodies described above, i.e., anti-human cellular TN polyclonal primary antibody (Gibco-BRL) for porcine tissue and anti-rat formaldehyde-fixed TN polyclonal antibody (1:100) for rat tissue. Endogenous peroxidase activity was blocked by immersing the sections in methanol with 1% hydrogen peroxide and nonspecific binding was blocked using 5% normal goat serum. Sections were
incubated in primary antibody (1:50 dilution) overnight at 4°C and antibody binding was visualized using the Vectastain ABC System (Vector Laboratories, Burlingame, CA) and developed with 3,3’-diaminobenzidine (DAB) (Sigma). Control sections were treated with normal rabbit isotypic IgG (Dako Corp., Glostrup, Denmark). Nuclei were counterstained using haematoxylin.

The relative abundance of TN in the porcine sections examined was graded quantitatively in 5 random fields (290 µm² fields at an original magnification of 400x) per sample using the Image-Pro Plus program for Macintosh (Media Cybernetics, Silver Spring, MD). The program determines a uniform cut-off considered 'background' and will both planimeterize and perform densitometry on the positively immunostained sections. Subsequent multiplication of both the total area positively stained and the average density of staining provides a relative densitometric unit of TN deposition which was then averaged between fields and means calculated for each tissue sample.

Proliferating cells were identified by detecting proliferating cell nuclear antigen (PCNA), a 37kD protein that is present in proliferating cells where it complexes with DNA polymerase δ to enable chain elongation. Endogenous peroxidase activity was blocked by immersing the sections in methanol with 1% H₂O₂ followed by a nuclease digestion (antigen retrieval) and incubation with the anti-PCNA monoclonal primary antibody (1:100) (Dako). Control sections were incubated with normal mouse isotypic IgG (Dako) and a normal human skin section was used as a positive control. Antibody binding was visualized using 3,3’-diaminobenzidine (Sigma) and a substrate intensifier (Amersham International, Little Chalfont, UK). Cytoplasmic counterstaining was performed using eosin.

The presence of cellular apoptosis was determined using the Apoptag in situ detection system (Oncor Inc., Gaithersberg, MD). Briefly, paraffin sections were deparaffinized,
rehydrated, washed in PBS, and digested with 20 μg/ml proteinase K at room temperature for 15 minutes. Samples were placed in ethanol and acetate (2:1) at -20°C for 5 minutes followed by exposure to equilibration buffer (Oncor) and incubation with terminal deoxynucleotidal transferase enzyme (Oncor) at 37°C for one hour to facilitate the addition of digoxigenin labeled nucleotides to the 'exposed' 3'-OH ends of fragmented DNA (a characteristic feature of cellular apoptosis). Samples were then immersed in pre-warmed stop wash buffer (Oncor), incubated for 30 minutes at room temperature with anti-digoxigenin antibody. On porcine sections, the secondary antibody was peroxidase-conjugated whereas we used a fluorescein-conjugated secondary antibody on rat sections. A normal skin sample was used as a positive control while a section incubated in the absence of primary antibody was used as a negative control. Nuclear counterstaining was performed on porcine sections with methylene green and on rat sections with propidium iodide.

The presence of proliferating and apoptotic cells was also quantitatively assessed in ten randomly selected fields (290 μm² fields at an original magnification of 400x). The number of positive cells and the total number of cells (nuclei identified by propidium iodide counterstaining) per field was counted, a percent positive cells calculated, and a mean percent generated for each treatment condition.

**Detection of Necrosis**

Cellular necrosis was assessed as membrane damage resulting in the leakage of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the extracellular space. LDH, which converts lactate and NAD⁺ to pyruvate and NADH, was assayed by providing the substrate for this enzyme and subsequently quantifying the amount of enzyme released as a function of NAD⁺ converted (LDH Release Assay Kit; Sigma). The relative abundance of LDH within a given sample is assessed following a series of oxidation-reduction reactions
resulting in the production of a purple precipitate (tetranitroblue tetrazolium formazen) which can be measured spectrophotometrically. 120 µl of conditioned medium was incubated in the dark with 30 µl of a 1:1 mixture of substrate and buffer (Sigma). The resultant colour change was measured on a plate reader at a wavelength of 490 nm. To account for any background absorbance or LDH activity in the culture medium, both serum free medium and medium containing 5% fetal bovine serum were used as negative controls and subtracted from the experimental measurements. To ensure that the assay was sensitive enough in detecting LDH released into the medium, supernatants from PA SMCs following freeze-thaw cycles were used as positive controls.

**Statistical Analysis**

Experiments were performed at least 3 times (exact numbers are given in the Figure legends) and values obtained at each time point were expressed as mean±SEM. Statistical significance between groups was determined using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (Fisher's LSD) test of multiple comparisons to establish differences between individual groups.
RESULTS

Matrix Metalloproteinase Activity in Porcine Pulmonary Arteries

Since MMP-2 regulates TN deposition in cultured PA SMCs (Jones, et al., 1997b; Jones, et al., 1999), net MMP activity was assessed in PA tissue extracts by gelatin zymography and reverse zymography. Gelatinolytic bands at ~83kD and a triplet at ~52, 56, and 60kD were detected (Fig. 2.1) and confirmed to result from MMP activity through their abrogation following incubation in EDTA (data not shown). Furthermore, some of these bands were characterized by native western immunoblotting to be MMP-9 (83kD), and the active and latent form of MMP-2 respectively (52 and 56 kD) (data not shown). The enzyme responsible for the 60kD gelatinolytic band was not identified. Qualitative analysis of the zymograms showed a reproducible increase in MMP-2 activity in attached cultures on day 5 compared with day 0. This increase in MMP-2 was evident in both attached and floating cultures by day 8, however, in attached cultures, but not in floating cultures, an increase in MMP-9 was now observed. These differences were confirmed as significant (p<0.05) by densitometric analysis, the interpretation of which is limited by the non-linear nature of the assay (data not shown).

Reverse gelatin zymography was performed to detect changes in native MMP inhibitors which would influence net MMP activity. Multiple bands corresponding to bound and free native inhibitors of MMPs were identified (data not shown). Western immunoblots for tissue inhibitor of MMP -1 (TIMP-1) were performed, identifying anti-gelatinolytic bands containing TIMP-1. However, no significant difference between attached and floating cultures was detected in any of the bands (data not shown).

Tenascin-C Protein Deposition in Porcine Pulmonary Arteries

Western immunoblotting and immunohistochemistry for TN was performed to determine whether attached versus floating culture conditions affect temporal and spatial deposition of
TN. On western immunoblot, TN was identified as a ~220 kD immunoreactive band that progressively increased in tissue cultured on attached collagen (Fig. 2.2). On day 5 there was a significant increase in both attached and floating gels relative to day 0 (p<0.05) but on day 8, values in floating cultures had fallen to control levels. There was therefore a >2 fold increase in TN on attached relative to floating PA organ culture (p<0.05).

Immunolocalization of TN revealed focal rather than homogenous deposition (Fig. 2.3A and B). We therefore developed a semi-quantitative method to evaluate the sections to take this into account. Planimeterization and densitometry of TN immunopositive regions revealed an increase in TN on attached collagen gels significant by day 5 (p<0.05) (Fig. 2.3E), while PAs on floating collagen displayed low levels of immunostaining. The decrease in TN immunostaining preceded the decrease in TN on western immunoblot suggesting either a difference in the sensitivity of the methods or an initial conformational change in TN in PAs on floating collagen gels making it less immunodense on tissue sections.

**Tenascin, Proliferating Cells and Apoptosis**

To assess whether TN deposition correlated with proliferating cells, immunostaining for PCNA was used while apoptosis was detected by in situ TUNEL assays. TN, accumulating in foci (Fig. 2.3A & B), co-localized directly with PCNA positive cells (Fig. 2.3C), and inversely with apoptotic cells (Fig. 2.3D). Planimetry and densitometry of TN rich and poor foci further confirmed this association as regions with high TN deposition had a similarly high proliferation index and low levels of apoptosis, while areas with reduced TN exhibited almost absence of proliferation and had a very high percent of apoptotic cells (Fig. 2.3F). Despite these correlations, changes in wall thickness were not observed when comparing attached and floating vessels (data not shown). If we divide TN staining into either negative, minimal, moderate, and intense, judged by relative densitometric units <0.01,
0.01-1, 1-10, and >10 (Fig. 2.3F), it is conceivable that floating cultures were composed of negative and moderate patches of TN and attached cultures were composed of minimal and intense patches. Thus, in both cultures proliferating and apoptotic cells were balanced (supported by total counts of PCNA positive and apoptotic cells, data not shown).

**Progression and Regression of Hypertrophied Rat Pulmonary Arteries**

Recent studies suggest that actively remodeling vessels are uniquely dependent on survival signals provided through interaction with the ECM (Brooks, et al., 1994a). Consequently, we investigated whether hypertensive vessels, already in a state of active remodeling, may be more responsive to manipulation of TN by attached versus floating conditions. Following MCT-treatment, a 30% increase in medial hypertrophy was observed when compared to saline controls (p<0.05) (Fig. 2.4A, B & E). This hypertrophy was not only perpetuated on attached collagen gels but there was a further 25% increase by day 8 (p<0.05) (Fig. 2.4C & E), while a progressive regression of medial hypertrophy over this time frame was observed on floating collagen (40% decrease over day 0) (p<0.05) (Fig. 2.4D & E). This progression and regression was not recapitulated by rat saline control vessels cultured in a similar fashion (data not shown) and indicated that normotensive rat PAs behave like the porcine PAs described previously in detail.

Progressive hypertrophy on attached gels was associated with a trend toward an increase in the number of elastic laminae (Fig. 2.4A, C & F) and deposition of elastin (data not shown). Regression of medial hypertrophy in floating cultures was associated with resorption of elastin (Fig. 2.4A & D), to values comparable to those in saline control vessels in terms of number of laminae (Fig. 2.4F) and elastin densitometry (data not shown).
Elastase and Matrix Metalloproteinase-2 Activity

Since serine elastases are increased in hypertensive PAs (Zhu, et al., 1994; Thompson, et al., 1998), they may play a direct role in the upregulation of TN through the activation of MMPs (Okada, et al., 1988; Okada and Nakanishi, 1989). Thus, elastolytic activity was examined by elastase assays comparing attached and floating cultures at 8 days. There was a 60% increase in elastin degrading activity in attached versus floating cultures (p<0.05) (Fig. 2.5A). To determine whether differences in MMPs exist in hypertensive PAs on attached and floating collagen gels, gelatin zymography was performed on tissues harvested at day 8 (Fig. 2.5B). A predominant gelatinolytic doublet was observed at ~56 and 52kD. Western immunoblotting of a similar native gel identified these bands as the latent and active form of MMP-2, respectively (not shown); on a reducing and denaturing gel these forms of MMP-2 migrate as ~72 and 66kD species (data not shown). A decrease in the active form of the enzyme was evident by either detection method in floating relative to attached cultures (83% by western immunoblot) (p<0.05), while both conditions retained similar amounts of latent enzyme. Release of MMPs into the culture media was also assessed. Only latent MMP-2 was detected by zymography in conditioned medium from attached and floating cultures and in similar amounts. The active form of MMP-2 was not observed (data not shown).

Deposition of Tenascin-C

Tenascin-C deposition, assessed by immunohistochemistry, was largely negative in PAs from saline injected rats (Fig. 2.6B). TN accumulated following MCT injection and was deposited throughout the vessel wall in a cell-associated fashion (Fig. 2.6A). TN deposition is further enhanced on attached collagen (Fig. 2.6C), in contrast to floating cultures where TN is suppressed (Fig. 2.6D). The marked reduction of TN in floating cultures was consistent with northern and western immunoblotting for TN (Fig. 2.6E and F). There was a >50% decrease in total TN mRNA, identified as ~7.3 and 6.4 kb alternatively spliced
isoforms, with a loss of TN immunoreactive bands (~230, 220 & 180kD) in three independent experiments. Extrusion of TN from these cultures, examined by immunoblotting conditioned medium, was not evident.

**Proliferation and Apoptosis**

Proliferating cell nuclear antigen immunostaining was performed and showed a significant induction of proliferation in PAs from MCT injected rats at 21 days compared to saline injected controls (Fig. 2.7). The number of medial proliferating cells progressively increased on attached collagen (>2 fold by day 8) (p<0.05), while PAs cultured on floating collagen showed minimal evidence of proliferation with PCNA values similar to saline controls (p<0.05).

We next determined, by performing TUNEL assays, whether apoptosis was related to the regression in medial hypertrophy and suppression of TN in these hypertrophied rat PAs when floated on collagen gels (Fig. 2.8A-E). To confirm that the *in situ* TUNEL assays were not aberrantly identifying necrotic cells as apoptotic, lactate dehydrogenase assays were performed. No significant difference was detected between attached and floating cultures and the tissue free control (data not shown), indicating that the observed vascular regression was not due to the onset of necrosis. Normalized counts of medial apoptotic cells revealed low levels of apoptosis in MCT and saline-injected rat PAs (Fig. 2.8A, B & E). When MCT injected rat PAs were cultured on attached collagen there was minimal or absent apoptosis (Fig. 2.8C & E). Conversely, floating cultures displayed an early and sustained >6 fold induction of medial apoptosis from day 3 (p<0.05) (Fig. 2.8D & E). In addition, normotensive rat PAs (saline injected) did not show an induction of apoptosis on either attached or floating gels (data not shown).
Figure 2.1

A representative gelatin zymogram of porcine PA organ culture tissue collected from attached and floating cultures at 0, 1, 3, 5, and 8 days is shown. Bands at ~83, 56 and 52kD reflecting MMP-9 and MMP-2 (active and latent form) respectively are evident in both attached (Att) and floating (Fl) conditions. ## S = molecular weight standards.
Figure 2.2

A depicts a representative western immunoblot for TN in porcine PA organ culture tissue collected from attached and floating cultures at 0, 1, 3, 5, and 8 days, and B reflects densitometric analyses from 3 different experiments. TN was identified in these tissues as a ~220kD immunoreactive band which increased in attached cultures and, in contrast, was reduced in floating cultures. Bars = mean + SEM of n = 3 vessels at each timepoint, * = p<0.05 comparing attached and floating and † = p<0.05 compared to day 0.
Figure 2.3

In this figure, photomicrographs of porcine PA organ culture tissue sections, while taken from an attached culture, are representative of samples collected from attached and floating cultures at 8 days and immunostained for TN (A & B), PCNA (C), and following TUNEL assay (E). Panel A is at a magnification of 400x, while panels B, C, and D are at 100x. In B, C, and D the boundary between a TN rich and TN poor region is illustrated on serially stained sections. C and D show that PCNA positivity (C) co-distributes with the TN rich area and apoptotic cells (D) with the TN poor area. Bar = 25 μm. Planimetric and densitometric grading of TN from porcine attached and floating cultures from day 0-8 is shown in E and counts of proliferating or apoptotic cells calculated from numerous TN rich and poor regions as described in the Methods is illustrated in F. Bars = mean + SEM of n = 3 vessels at each timepoint (n = 8 for F), * = p<0.05 comparing attached and floating and † = p<0.05 compared to day 0.
Figure 2.4

Representative photomicrographs of Movat stained rat PA organ culture tissue collected from attached and floating cultures at 0 and 8 days. Rat PAs taken at 21 days, the point at which they would be placed into culture, show medial hypertrophy (A), in contrast, to PAs from saline injected rats (B). On attached collagen gels, these vessels continue to thicken over the 8 days (C & E). Vessels on floating gels, however, show a progressive regression of medial thickness over the 8 days (D & E). Bar = 25 µm. Measurements of medial hypertrophy (E) showed significant corresponding differences and are associated with modulation of matrix, represented as increases in elastin on attached cultures and decreases in floating cultures. This is assessed by the number of elastic laminae (F) and by relative densitometric units of elastin in the vessel wall (data not shown). Bars = mean ± SEM of n = 3 vessels at each timepoint (n = 8 for graph of medial thickness), * = p<0.05 comparing attached and floating conditions, and † = p<0.05 compared to day 0.
Figure 2.5

Elastase assays were performed on MCT injected rat PAs after 8 days in organ culture and showed reduced elastolytic activity in floating versus attached conditions (A). A decrease in proteolytic activity in floating cultures was also observed following western immunoblotting for MMP-2 (not shown) and gelatin zymography (B). Densitometric quantification of differences in the active form of MMP-2 are shown in B; the amount of latent enzyme is similar in tissues on attached and floating gels. Bars = mean + SEM of n = 3 vessels, * = p<0.05 compared to attached conditions.
A

Total Elastolytic Activity (CPM)

<table>
<thead>
<tr>
<th>Attached</th>
<th>Floating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Pro-form 56kD
Active Form 52kD

Day 0 8 8

Attached Floating

<table>
<thead>
<tr>
<th>Relative Densitometric Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000</td>
</tr>
<tr>
<td>4000</td>
</tr>
<tr>
<td>3000</td>
</tr>
<tr>
<td>2000</td>
</tr>
<tr>
<td>1000</td>
</tr>
</tbody>
</table>

Pro-form of MMP-2
Active Form of MMP-2
Figure 2.6

Representative photomicrographs of TN immunohistochemistry in rat PA tissue reveal an increase in TN deposition 21 days following MCT injection (A) relative to saline injected controls (B). PA tissue harvested after 8 days in organ culture indicate an accumulation of TN in cultures on attached gels (C), while floating PAs reduce TN deposition such that its nearly absent at day 8 (D). Bar = 25 μm. A representative TN northern blot shows a decrease of total TN mRNA (~7.3 and 6.4 kb alternatively spliced transcripts observed) in tissue on floating compared to attached collagen gels, as compared to 28s rRNA loading control (E). A marked reduction in TN protein (~230, 220 and 180kD immunoreactive bands) was observed on western immunoblots (F). Bars = mean ± SEM of n = 3 vessels, * = p<0.05 compared to attached conditions.
Figure 2.7

Quantification of the percent of proliferating cell nuclear antigen (PCNA) positive cells in PA organ culture tissue on either attached or floating conditions as determined by immunohistochemistry. PCNA positivity is observed in PAs after MCT injection, but not from saline injected rats. Attached cultures show a progressive increase in positivity at days 3, 5 and 8, while floating cultures show reduced positivity on day 3 with PCNA staining being absent by days 5 and 8. Bars = mean + SEM of n = 3 vessels at each timepoint, * = p<0.05 comparing attached and floating and † = p<0.05 compared to day 0.
Apoptosis was monitored by TUNEL assay in PA organ culture tissue. In A and B, nuclei from the PAs of MCT (A) and saline control (B) injected rats after 21 days appear normal as stained by propidium iodide. There were few TUNEL positive cells in PA tissues on attached collagen gels in organ culture as shown on day 3 (C) and as quantified in E. With regression of medial thickness, apoptotic cells were identified as bright green fluorescent nuclei beginning on day 3 (D) and as quantified in E. Bar = 25 μm. Quantification of the percent apoptotic cells at each timepoint indicates that these observed differences were statistically significant (E). Bars = mean ± SEM of n = 3 vessels at each timepoint, * = p<0.05 comparing attached and floating and † = p<0.05 compared to day 0.
DISCUSSION

In this study we have shown that SMCs in the intact vessel, in the presence of endothelial cells, fibroblasts and surrounding ECM, respond to mechanical changes in collagen gels. When the vessels are maintained on attached collagen gels, MMPs and TN production, and cellular proliferation increase but, as a consequence of altered stress or deformation of the SMCs on floating gels, a reduction in MMPs and TN expression, and induction of apoptosis occur. The features described above are amplified when hypertrophied vessels are cultured in this fashion such that there is progression of medial wall thickening over time on attached collagen and regression on floating collagen gels. This model has extended observations related to cultured SMCs in collagen gels by revealing how coordinated loss of cellularity and matrix can structurally alter a blood vessel.

Aortic organ cultures have been previously used to study the behaviour of SMCs in their 'native' environment and to address factors that might lead to the development of a neointima (Gotlieb and Boden, 1984). These studies describing SMC neointimal proliferation together with fibroblast growth factor (FGF-2) expression (Daley and Gotlieb, 1996) and increased serine elastase activity (Oho, et al., 1995) have not, however, been applied to examine the evolution of structural changes in PAs. Unlike the porcine aorta, porcine or rat PAs do not have intimal SMCs, and this may explain why these vessels did not respond to organ culture by developing a neointima. However, the organ culture model is faithful to clinical and experimental observations related to TN expression and SMC proliferation in the pathophysiology of remodeling and confirm our cell culture observations, indicating the importance of MMP-2 activity. We now document that the 'reverse process' results in regression of medial hypertrophy.

Previous studies have shown that TN is upregulated in hypertensive rat arteries (Mackie, et al., 1992; Jones and Rabinovitch, 1996) and in response to angiotensin II (Mackie, et al.,
We have linked the changes in PA organ culture observed on attached and floating collagen gels to the effects of mechanical stress or deformation. Previous studies have shown that TN is regulated by alterations in mechanical stress imposed on cells through the use of the attached/floating collagen gel system (Chiquet-Ehrismann, et al., 1994). These conditions affect cell shape, which is well established as an important determinant of cellular function (Folkman and Moscona, 1978; Singhvi, et al., 1994; Chen, et al., 1997). Vascular cells are naturally poised to respond to mechanotransduced signals from the matrix (Singhvi, et al., 1994; Chen, et al., 1997) as a result of changes in haemodynamic factors (Topper, et al., 1996; Topper, et al., 1997). Studies in our laboratory have confirmed that vascular SMCs will respond like fibroblasts by upregulating TN on attached collagen gels (Chiquet-Ehrismann, et al., 1994; Jones, et al., 1997b). Here we show that the increase in TN by immunohistochemistry and immunoblot is associated with heightened mRNA levels. A putative 'stress response sequence' in the TN promoter was identified in fibroblasts (Chiquet-Ehrismann, et al., 1994), but does not appear to be the region in SMCs that is responsive in attached cultures (Jones, et al., 1999).

In these organ culture studies, changes in TN expression were associated with MMP-2 and -9 activity and protein levels, being consistent with our previous studies showing that MMP-2 activity regulates TN synthesis in cultured SMCs (Jones, et al., 1997b). This mechanism which involves MMP-mediated degradation of collagen, increases TN transcription via a MAP kinase pathway activated following SMC ligation with cryptic RGD sites exposed in denatured collagen (Jones, et al., 1999). Conversely, inhibition of MMP-2 on floating collagen or by the MMP inhibitor GM-6001 was associated with a reduction in TN expression and onset of apoptosis (Jones, et al., 1997b). Thus, there is a functional relationship between TN and MMPs, which co-distribute at sites of vascular pathology (Zempo, et al., 1994; Jones and Rabinovitch, 1996; Patel, et al., 1996; Jones, et al., 1997a).
Since MMP-2 regulates TN on floating collagen gels, it remains to be established how induction of MMP-2 might be related to alterations in mechanical stress or deformation. It is possible that there are mecano-responsive elements regulating the gene for this enzyme or that MMP-2 is induced by serum factors, endothelial factors or cytokines which gain access to the subendothelium following mechanical perturbation of the endothelial surface. This has been proposed as a mechanism leading to the induction of elastase activity in cultured SMCs (Thompson, et al., 1998; Wigle, et al., 1998).

Our experiments using hypertrophied rat PAs documented a relative increase in elastase activity in cultures on attached versus floating collagen gels. As hypertensive vessels exhibit elevated serine elastase activity (Zhu, et al., 1994), these matrix proteinases might contribute to the pathogenesis of medial hypertrophy in organ culture through a TN-dependent pathway. Elastases may direct the upregulation of endogenous TN either directly, following growth factor liberation from matrix (Rettig, et al., 1994; Thompson and Rabinovitch, 1996), or indirectly through either MMP activation (Okada, et al., 1988; Okada and Nakanishi, 1989) or increased expression, by products of elastolysis (Werb, et al., 1989; Thompson and Rabinovitch, 1996; Tyagi, et al., 1996; Miyake, et al., 1997). This may account for the substantial amplification of differences in MMP-2 activity in attached and floating cultures when comparing hypertensive versus normotensive PAs.

The impact of MMP-modulation of TN on SMC proliferation has been shown in these organ culture studies and some of the mechanisms involved have previously been addressed in cell culture. TN acts as a critical SMC survival factor, which functionally amplifies the SMC proliferative response to liberated growth factors. Priming of growth factor receptors in this way occurs through their clustering at focal adhesion contacts formed by αvβ3 mediated interaction of SMCs with TN and cytoskeletal reorganization (Jones, et al., 1997b).
Our studies have also supported evidence showing that withdrawal of TN leads to apoptosis. This mechanism, largely unexplored, is less well understood, but may be related to the initiation of 'death gene' inductive intracellular signals following unmasking of integrins, particularly β₃, in the presence of growth factors (Meredith, et al., 1993; Brooks, et al., 1994b; Ruoslahti and Reed, 1994; Stromblad, et al., 1996). Evidence suggesting a requirement of SMCs for TN in actively remodeling vessels comes from work documenting the presence of TN during vasculogenesis (Kostianovsky, et al., 1997) and its reappearance during vascular injury (Jones and Rabinovitch, 1996) and angiogenesis (Zagzag, et al., 1995). Further evidence is suggested by recent work in which vascular cell survival and survival-related signals are mediated by ligation of the α₅β₃ integrin receptor (Stromblad, et al., 1996; Scatena, et al., 1998), which is the receptor for TN on SMCs (Prieto, et al., 1993; Jones, et al., 1997b). Indeed, αᵥ knockout mice show extensive vascular remodeling and embryonic lethality (Bader, et al., 1998). This profound response, together with reports that TN knockout mice exhibit only a very mild phenotype (Saga, et al., 1992; Fukamauchi, et al., 1996), suggests that this receptor may be critical for signaling of a number of alternative matricellular proteins. Recent unpublished data from our laboratory indicates that TN suppression within the vasculature is accompanied by upregulation of an alternative αᵥβ₃ ligating cell survival factor, osteopontin. While this may account for differences in embryonic development between these two mice, consistent with evidence that vessel formation is inhibited with an αᵥβ₃ functional blocking antibody, it is interesting that this response is selective, in that established vessels do not require survival signals provided by αᵥβ₃-matrix interaction (Brooks, et al., 1994b; Drake, et al., 1995). This may explain the amplification of the MMP-TN-mediated effect in hypertrophied PAs, as the SMCs in these remodeling vessels may be more dependent on β₃ integrin signals compared to normal PAs which receive viability signals through other receptors, like the β₁ integrins (Boudreau, et al., 1995; Zhang, et al., 1995; Jones, et al., 1997b).
Previous reports documenting a reduction in vascular wall thickness have focused solely on loss of cellularity (deBlois, et al., 1997; Pollman, et al., 1998), whereas a coordinated depletion of both cells and matrix is likely required for an optimal response. Progression of medial hypertrophy in PAs on attached gels was associated with an increase in the number of elastic laminae. It might be expected that an upregulation of matrix synthesis results from an increase in SMC cellularity and recruitment of resident SMCs to a phenotypically modulated, synthetic state. Our previous studies have also shown high turnover of elastin in association with progression of PA medial hypertrophy and have implicated increased elastase activity, consistent with our present findings (Zhu, et al., 1994; Thompson, et al., 1998). The mechanism appears to be related to elastin peptide induction of elastin synthesis as has been demonstrated in fibroblast culture (Foster, et al., 1990).

Conversely, SMC apoptosis is associated with loss of elastin. Intriguing was that the decrease in elastin was associated with a reduction in the number of elastic lamina. This process may represent the preferential loss of more-susceptible, newly synthesized elastic lamina (Stone, et al., 1987; Stone, et al., 1988), given that the number of lamina returned to match saline levels. Also of particular interest is that the ECM resorption was associated with suppression of classical matrix degrading enzymes, MMP-2 and elastases. In the regression of medial hypertrophy that occurs in rat PAs following removal of animals from a hypoxic environment, increased expression of collagenase in mast cells has been reported (Tozzi, et al., 1998), but a cause and effect relationship has not been determined. Since loss of elastin occurs under conditions of reduced proteinase activity in our model, we speculate, based on studies with monkey gingival fibroblasts (Sawada and Inoue, 1997), that elastin is being phagocytosed by vascular cells and degraded intracellularly along with other matrix constituents. Alternatively, during the process of apoptosis, membrane permeability
changes (Ormerod, et al., 1993; Lang, et al., 1998) may result in activation of cell surface enzymes which proteolyze the ECM in the microenvironment.

In conclusion, we present a model whereby diseased PAs, which progressively hypertrophy on attached collagen, selectively undergo vascular regression on floating gels related to a reduction in proteolytic activity of both MMP-2 and elastases, downregulation of TN, suppression of proliferation, and induction of apoptosis. We suggest that progression versus regression of PVD is consequently dependent on appropriate perturbation of fundamental cell-matrix interactions. Effective vascular lesion regression involves depletion of both ECM and cells, and a therapeutic strategy should ideally be directed at both these components of the vessel wall.
CHAPTER THREE

Elastase and Matrix Metalloproteinase Inhibitors Induce Regression and Tenascin-C Antisense Prevents Progressive Vascular Disease
ACKNOWLEDGEMENTS

I would like to acknowledge the contribution of Dr. Peter L. Jones. During the course of the study presented in this chapter, Dr. Jones provided a critical assessment of samples and data as well as contributing to experimental planning.

INTRODUCTION

Pulmonary hypertension results from a variety of initiating stimuli. Its progression is associated with abnormal endothelial morphology and function (Rabinovitch, 1998), muscularization of normally non-muscular peripheral arteries related to differentiation of pericytes (Meyrick and Reid, 1980b), and medial hypertrophy and neointimal formation in muscular arteries, as a consequence of hypertrophy, proliferation, and migration of resident smooth muscle cells (SMCs) and increased production of extracellular matrix (ECM) components. These include collagen, elastin, fibronectin and tenascin-C (TN) (Prosser, et al., 1989; Jones, et al., 1997a; Rabinovitch, 1998). Experimental models have successfully recapitulated many of these features (Rabinovitch, et al., 1986; Prosser, et al., 1989). We have provided evidence that endothelial alterations may allow extravasation of a serum factor capable of stimulating SMC production of a vascular serine elastase (Todorovich-Hunter, et al., 1992; Zhu, et al., 1994; Thompson, et al., 1998; Wigle, et al., 1998). In cultured SMCs, a consequence of elastase-mediated degradation of ECM includes the liberation of matrix bound SMC mitogens, such as basic fibroblast growth factor (FGF-2) (Todorovich-Hunter, et al., 1992; Oho, et al., 1995; Thompson and Rabinovitch, 1996). This serine elastase, if similar to others such as leukocyte elastase, may also direct further matrix degradation in disease through the activation (Okada and Nakanishi, 1989; Itoh and Nagase, 1995) and/or expression (Landeau, et al., 1994; Thompson and Rabinovitch, 1996; Miyake, et al., 1997; Thompson, et al., 1998) of matrix metalloproteinases (MMPs) whose presence has been documented at sites of vascular remodeling (Zempo, et al., 1994; Patel, et al., 1996).
Increased MMP activity in cultured SMCs induces expression of the ECM glycoprotein TN (Jones, et al., 1997b) which has been described in vascular diseases (Hedin, et al., 1991) including clinical and experimental pulmonary hypertension (Jones and Rabinovitch, 1996; Jones, et al., 1997a). Evaluation of the pathophysiologic role of TN in pulmonary hypertension revealed a relationship with SMC proliferation. Both in monocrotaline (MCT)-induced hypertensive PAs and in PAs from patients with congenital heart disease, a progressive increase in TN expression correlated with proliferating SMCs (Jones and Rabinovitch, 1996; Jones, et al., 1997a). Subsequent studies in cultured PA SMCs documented that TN amplifies the mitogenic response to FGF-2 and is a prerequisite for epidermal growth factor (EGF)-dependent SMC proliferation (Jones and Rabinovitch, 1996).

Further studies related the cellular mechanisms underlying MMP-dependent TN gene transcription to β3 integrin ligation and induction of MAP kinase activity (Jones, et al., 1999). TN-mediated SMC proliferation resulted from clustering of its cognate (αvβ3 integrin) receptors, leading to formation of specialized focal adhesions and consequent clustering and phosphorylation of growth factor receptors (Jones, et al., 1997b). Conversely, when MMPs are inhibited either directly or by culturing SMCs on floating collagen gels, endogenous TN levels are markedly reduced and SMC apoptosis is evident. Recent studies carried out in organ culture confirmed that SMCs in the hypertrophied PA responded similarly in attached and floating collagen gels and established that changes in elastase activity correlate with MMP activity (Cowan, et al., 1999).

We now show that direct inhibition of MMPs, including gelatinolytic activity attributable to MMP-2, or of serine elastases leads to reduced TN, induction of apoptosis, loss of ECM and regression of PA hypertrophy. Transfection of a TN antisense/ribozyme expression vector reduced TN mRNA and protein and resulted in considerable SMC apoptosis.
although proliferation also occurred. This arrested progressive hypertrophy, but failed to induce regression. Immunohistochemistry revealed an increase in osteopontin (OPN), an alternative \(\alpha_\nu\beta_3\) ligand, possibly in response to cues provided by a proteolytic environment. We confirm that OPN functions as a survival factor rescuing SMCs from MMP-inhibitor induced apoptosis and that \(\alpha_\nu\beta_3\) blockade can promote SMC apoptosis.
MATERIALS AND METHODS

Preparation of Explants

PAS were harvested from adult male Sprague-Dawley rats (300g) (Charles River Laboratories, St. Laurent, CN) 21 days following a subcutaneous injection of saline or the alkaloid toxin monocrotaline (60mg/kg) (MCT) (Sigma Chemical Co., St. Louis, MO) in accordance with protocols approved by the Animal Care Committee of The Hospital for Sick Children. The rats were sacrificed, main PAS excised, and cultured in attached type I collagen gels as previously described (Cowan, et al., 1999).

Experimental Designs

In the first set of experiments, organ cultures were supplemented with one of the following 3 proteinase inhibitors or with their administration vehicles. The general serine elastase inhibitor alpha1-proteinase inhibitor (α1-PI) (6mg/ml) (endotoxin free Prolastin, a kind gift from Ms. M.A. Lark, Cutter Biologicals, Miles, Etobicoke, ON), in PBS, the synthetic MMP inhibitor GM-6001 (8μM) (a kind gift from Dr. S. Sweidler, Glycomed, Alameda, CA) dissolved in 0.4% dimethyl sulphoxide (DMSO) (Calbiochem-Novabiochem Corp., La Jolla, CA) or the selective neutrophil elastase inhibitor 1K (ZD0892) (0.8μM) (a kind gift from Dr. C. Veale, Zeneca Pharmaceuticals, Wilmington, DW), delivered in 5% polyethylene glycol (PEG)(Sigma). Inhibitors were chosen to target MMPs or the heightened elastase activity demonstrated in MCT-induced vascular disease (Todorovich-Hunter, et al., 1992; Jones, et al., 1997b; Cowan, et al., 1999; Jones, et al., 1999). Doses were based upon our previous studies (Oho, et al., 1995; Jones, et al., 1997b; Lee, et al., 1998).

α1-PI is a broad spectrum serine proteinase inhibitor (Beatty, et al., 1980). GM-6001, an N-[2(R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide, is a non-cytotoxic, synthetic inhibitor that functionally and specifically inhibits MMP
activity by complexing with the zinc atom found in the active site of MMPs, and prevents substrate interaction, as described (Jones, et al., 1997b). 1K (ZD0892) is a peptidly trifluoromethyl ketone with an N-terminal 4-(CH₃O)C₆H₄CO group making it an orally bioavailable serine elastase inhibitor (Edwards, et al., 1997). It is highly selective for neutrophil elastase (kᵢ=6.7nM), but also inhibits pancreatic elastase (kᵢ=200nM), and endogenous vascular elastase (unpublished data), but not metallo, cysteine or other serine proteinases. It is preferable to elafin which is relatively unstable and must be given by continuous administration (Cowan, et al., 1997).

**Preparation of Sense and Antisense Vectors for Transfection**

A U1Bam pZeo mut Eco RI/ Spe I expression vector backbone was a gift from Drs. H.C. Dietz and R.A. Montgomery (Johns Hopkins, Baltimore MD) (Montgomery and Dietz, 1997). Ligated into this plasmid, downstream from a constitutively active U1 promoter and between two snRNA hairpin loops, was a 32 bp antisense construct directed towards the 6th fibronectin type III constant domain of rat TN (coding sequence 5'-GAAAATGCAGCCACA .... TCAGCTGGAGGCACCG-3') and interrupted in its middle by a 22 bp sequence (relative to TN coding sequence 5'-CAAAGCAGGAGTGCTGAGTAG-3') encoding a hammerhead ribozyme. Antisense target sequence selection involved maintenance of hairpin loop 2" structures and targeting of the ribozyme to mRNA containing a 'GUC' ribozyme consensus cleavage site (Fig 3.1). Folding of the resultant cRNA activates the encoded ribozyme, such that binding of the construct to the target TN mRNA results in cleavage of the TN mRNA at the consensus cleavage site, destabilizing it and leading to its degradation and loss by endogenous exonuclease activity. The cRNA produced by the vector is particularly stable due to the presence of the 5' and 3' hairpin loops which confer resistance to exonuclease activity as a result of their high G+C content and facilitate nuclear trafficking of the construct due to lack of polyadenylation and the presence of a 5'trimethyl guanosine cap. Sense and
antisense oligonucleotides containing alternative 4 bp Eco RI and Spe I overhangs (antisense: 5' -CTAG GAA AAT GCA GCC ACA GTT TCG TCC TCA CGG ACT CAT CAG AGC TGG AGG GCA CCG; sense: 5'-AA7T CGG TGC CCT CCA GCT CTG ATG AGT CCG TGA GGA CGA AAC TGT GGC TGC ATT TTC) were denatured, annealed, and ligated into the Eco RI and Spe I (Pharmacia Biotech Sverige, Uppsala, Sweden) linearized expression vector using T4 DNA ligase (Gibco).

Prior to transfection, plasmid DNA was linearized with Apa I (Pharmacia) and bound to the non-histone associated nuclear proteins, high mobility group (HMG) -1 and -2 (Wako Pure Chemical Industries), to facilitate nuclear targeting of the plasmid DNA (Yonemitsu, et al., 1996). The plasmid/HMG complex was enclosed in liposomes and bound with UV-inactivated HVJ (a kind gift from Drs. Y. Kaneda and N. Nakamura, Osaka University, Japan).

PAS were excised and both infused and incubated for 10 minutes in 250μl of buffered saline containing 2μM of CaCl₂ and 7,500 haemagglutinating units of HVJ bound to liposomes containing 50μg of TN sense or antisense plasmid DNA or the constitutively active reporter expression plasmid pECE-CAT encoding full length chloramphenicol acetyltransferase (CAT).

αvβ3 Integrin Blocking

Hypertrophied PAS were cultured in media supplemented with either 15μg/ml of an anti-αvβ3 functional blocking antibody, LM609 (Chemicon, Temecula, CA), or 15μg/ml of control IgG (PharMingen, San Diego, CA) as previously described for cell culture (Jones, et al., 1997b).
**Cell Culture**

Vascular SMCs were isolated from the excised PAs of Sprague-Dawley rats and confluent cultures were collected by trypsinization and seeded on prepared collagen gels as previously described (Jones, et al., 1997b). Briefly, SMCs were cultured for 48 hours in medium 199 (Sigma) with 0.1% bovine serum albumin and 50ng/ml of epidermal growth factor (Gibco) and on gels containing either collagen alone or supplemented with 100nM of recombinant wild-type OPN expressed in E. coli as a glutathione S-transferase fusion protein (a kind gift from Dr. C.M. Giachelli, University of Washington, Seattle, WA) (Scatena, et al., 1998). Cell number was determined following collagenase digestion and counts were made using an improved Neubauer hemocytometer (American Optical, Buffalo, NY). Survival was quantified as a percent of SMCs present at 48 hours relative to the number of seeded cells (cells plated - cells in medium).

**Elastase Assays, and Matrix Metalloproteinase Activity and Expression**

The ability of conditioned media to degrade 200μg of [3H]-elastin was determined as previously described (Cowan, et al., 1999). To control for non-enzymatic degradation, radiolabeled elastin was incubated with medium cultured in the absence of tissue. Activity was related to a standard curve generated with human leukocyte elastase (0.075-5.0ng) (Elastin Products Comp.). Matrix metalloproteinase zymography was performed following homogenization of PA tissue as previously described (Cowan, et al., 1999), normalized by Bradford assay to total protein (Bio-Rad Laboratories, Hercules, CA). MMP western immunoblotting used an anti-MMP-2 rabbit polyclonal antisera (a kind gift from Drs. M. Silverman and M. Ailenburg, University of Toronto, Canada) as previously described (Cowan, et al., 1999).
Northern Blotting for Tenascin-C

Northern blot analysis was performed on 8 μgs of total RNA extracted from 3 pooled PAs and hybridized to a 250-bp cDNA probe derived from the seventh fibronectin type III constant domain of rat TN (Cowan, et al., 1999). TN mRNA was corrected for loading conditions by direct comparison with 28S rRNA, detected after ethidium bromide staining, and GAPDH, following hybridization with a rat specific probe.

Immunohistochemistry and Detection of Apoptosis

Immunohistochemistry, using techniques previously described (Cowan, et al., 1999), identified TN with an anti-rat formaldehyde-fixed TN polyclonal antibody (1:100) (a generous gift from Dr. H. Erickson, Duke University Medical Center, Durham, NC). For detection of PCNA, an anti-PCNA monoclonal antibody (1:100) (Dako) was used. Immunohistochemistry for OPN was performed with a monoclonal antibody raised against rat bone OPN, clone MPIIB101 (11ng/ml) (Department of Biological Sciences, University of Iowa, Iowa City, IA). Fibronectin (FN) was detected using an anti-FN monoclonal antibody (1:100) (Chemicon). For CAT immunostaining, an anti-CAT polyclonal antibody (1:50) (5 Prime - 3 Prime Inc., Boulder, CO) was used. Estimates of total elastin and collagen were detected using the Movat pentachrome stain. To quantify apoptosis, TUNEL assays were performed with the Apoptag in situ detection system (Oncor Inc., Gaithersberg, MD). Nuclear morphology was examined by labeling with propidium iodide (2μg/ml). DNA laddering was performed as previously described (Jones, et al., 1997b). In the cell culture studies, apoptosis was confirmed as the mechanism for reduced cell number using the DePsipher detection system (R&D Systems, Minneapolis, MN) which indicates loss of mitochondrial membrane potential. This assay correlates well with TUNEL and can be expediently applied to cells in culture but not readily to whole tissue samples.
The relative abundance of the ECM components examined were graded quantitatively, in 5 random fields (400x) per sample, using the Image-Pro Plus program for Macintosh (Media Cybernetics, Silver Spring, MD). The program performs planimetry and densitometry on positive staining above a uniform, 'background' cut-off. Subsequent multiplication of both the total area positively stained and the average density of staining provides a relative densitometric unit of ECM protein content which was then averaged and tissue means calculated. The relative number of proliferating and apoptotic cells was quantitatively assessed in 10 randomly selected fields (400x) as a percent of total propidium iodide stained cells.

**Lactate Dehydrogenase Assay**

Necrosis was assessed by a lactate dehydrogenase (LDH) release assays kit (Sigma). Controls included tissue-free medium cultured with and without FBS, as well as supernatants from PA SMCs following freeze-thaw cycles.

**Morphometric Analysis of Movat Pentachrome Stained Sections**

Twenty-five measurements of medial thickness, from internal to external elastic lamina, were recorded over the length of each vessel and means calculated.

**Statistical Analysis**

Values from multiple experiments are expressed as mean±standard error and statistical significance was determined using one-way analysis of variance followed by Fisher's least significant difference test of multiple comparisons to establish differences between individual groups. The number of samples in each group is indicated in the Figure legends.
RESULTS

Serine Elastase and Matrix Metalloproteinase Inhibition and TN Deposition

Tenascin-C is upregulated in SMCs cultured on proteolyzed collagen and suppressed following inhibition of MMP activity (Jones, et al., 1997b). Increased activity of a 20kD serine elastase (Zhu, et al., 1994) and MMP-2 (Cowan, et al., 1999) have been observed in PAs following MCT-injection. We therefore addressed the effects of inhibitors of serine elastases including endogenous vascular elastase, or of MMPs, on TN expression in rat PAs 21 days after MCT-injection and following 8 days in organ culture. Degradation of [3H]-elastin in conditioned medium from PA organ cultures was reduced in experiments with α1-PI, 1K and the MMP inhibitor GM-6001 compared to their vehicle controls (p<0.05) (Fig 3.2A). While the exact nature of the enzyme in the PA responsible for the elastase activity has not been identified, it is likely the endogenous serine elastase previously described in the hypertrophied PA after MCT injection, which would be inhibited by both α1-PI and the more selective agent, 1K. However, GM-6001 also inhibits the elastolytic activity suggesting either that there is an equally important MMP elastolytic component, or that MMPs regulate serine elastases, possibly by inactivating inhibitors (Mast, et al., 1991). The elastolytic activity measured is relatively small but it may only reflect the fact that most of the secreted elastases would be bound by inhibitors such as α2-macroglobulin or α1-PI or TIMPs which are present in the serum component of the conditioned media.

Gelatin zymography for MMP activity using tissue extracts from the same PA organ cultures detected multiple gelatinolytic bands, the most prominent of which was a doublet at ~56 and 50kD displayed in figure 3.2B. This gelatinolytic doublet corresponded to MMP activity since it was suppressed by the zinc/calcium chelator EDTA and was identified by western immunoblotting of a similar native gel to be the latent and active form of MMP-2 (data not shown). An approximate 3 fold suppression in MMP-2 activity was observed with serine elastase or MMP inhibitors compared to vehicle-treated controls (Fig 3.2B)
The suppression of latent MMP-2 could result from proteinase inhibition, since there would be a reduction in the ECM degradation products which normally stimulate transcription of the enzyme (Werb, et al., 1989; Huhtala, et al., 1995). While we focused on MMP-2 based on previous studies (Jones, et al., 1997b; Cowan, et al., 1999), additional, less prominent bands were also inhibited with GM-6001 and/or EDTA, suggesting the possible involvement of other MMPs. In addition, the inhibitory profile of GM-6001 may include members of the ADAM family of proteinases (Amour, et al., 1998). Taken together, our organ culture data suggest suppression of elastases and MMPs in an interdependent manner.

Deposition and localization of TN within PAs was documented by immunohistochemistry. TN accumulation was increased in association with MCT-induced PA hypertrophy (Jones and Rabinovitch, 1996), and deposited throughout the vessel wall (Fig 3.3A, relative to saline control Fig 3.3B). This deposition pattern was maintained or enhanced over 8 days in organ culture in the presence of the administration vehicles for the inhibitors, PBS (Fig 3.3C), DMSO (Fig 3.3E), and PEG (Fig 3.3G). Proteinase inhibition by α1-PI (Fig 3.3D), GM-6001 (Fig 3.3F), and 1K (Fig 3.3H) markedly reduced TN accumulation such that, with GM-6001 and 1K in particular, TN accumulation was severely limited. This differential deposition was confirmed by semi-quantitative grading (data not shown).

**Proteinase Inhibition and SMC Apoptosis**

Previous cell culture studies have shown that suppression of TN induces SMC apoptosis (Jones, et al., 1997b). To provide both quantification of the number of apoptotic cells and their tissue distribution, in situ TUNEL assays were performed on PAs after 8 days of organ culture in the presence and absence of the various inhibitors (Fig 3.4A-H and 3.5A). Less than 5% of cells were TUNEL positive at the time of organ culture, both in PAs harvested following either MCT (Fig 3.4A) or saline control (Fig 3.4B) injections. A
similar number of TUNEL positive cells was detected when vessels were cultured for 8 days with either PBS (Fig 3.4C), DMSO (Fig 3.4E), or PEG (Fig 3.4G) alone. α₁-PI (Fig 3.4D), GM-6001 (Fig 3.4F), and 1K (Fig 3.4H) all induced widespread TUNEL positivity primarily within the vessel media, but also in the adventitia and endothelium. The percent of these cells was quantified with α₁-PI (15%), GM-6001 or 1K (30% for both) and vehicle controls (~5-7%) (p<0.05) (Fig 3.5A). The appearance of TUNEL positive cells correlated with nuclear fragmentation and condensation. To determine whether the tissues were necrotic due to the cytotoxicity of the inhibitors, lactate dehydrogenase assays were performed but no enhanced activity was apparent compared to controls (data not shown).

To address the specificity of proteinase inhibition on the induction of apoptosis in MCT-treated hypertrophied PAs, we examined vessels from saline-injected rats in organ culture with media supplemented with proteinase inhibitors or their administration vehicles. TUNEL assays indicated that vascular cells from these vessels did not undergo apoptosis (Fig 3.5B).

**Proteinase Inhibition of PA Organ Cultures and SMC Proliferation**

Studies with PA SMC cultures on TN-supplemented collagen gels indicate that TN's role in disease may occur through an induction of growth factor responsiveness of these cells (Jones and Rabinovitch, 1996). To determine whether modulation of TN within a three dimensional native vessel configuration will affect the proliferative potential of vascular cells, we performed PCNA immunohistochemistry on cultured tissue (graphically depicted in Fig 3.5C; photomicrographs not shown) (p<0.05 for each comparison). Low levels of proliferating cells, judged by PCNA positivity (~3%), were present in PAs from rats 21 days following treatment with MCT (in contrast to saline controls where values were <1%). The further increase in PCNA positivity in organ culture was documented with vehicle-treated samples, either PBS (α₁-PI control) or PEG (1K control) (~12% for both), but not
with DMSO (GM-6001 control). A reduction of PCNA positivity occurred with all three inhibitors, α₁-PI, GM-6001, and 1K (p<0.05). Proteinase inhibitors do not influence proliferation in cultured control vessels from saline-injected rats as judged by PCNA immunostaining which is <1%.

**Proteinase Inhibitors and Regression of Vessel Wall Hypertrophy**

To determine whether regression of medial hypertrophy occurs as a result of proteinase inhibition and apoptosis, we measured PA wall thickness in Movat pentachrome stained tissue sections (Fig 3.6A-H & quantified in Fig 3.6I). Consistent with previous reports (Todorovich-Hunter, et al., 1992), MCT induced an increase in vessel wall thickness (Fig 3.6A & Fig 3.6I) compared to PAs derived from saline-injected rats (Fig 3.6B & Fig 3.6I). PAs cultured with administration vehicle controls continued to thicken (Fig 3.6C, PBS; E, DMSO; & G, PEG). In contrast, proteinase inhibitors showed regression of wall thickness to saline control levels with α₁-PI (Fig 3.6D), and to below those levels with GM-6001 (Fig 3.6F), and 1K (Fig 3.6H) (displayed graphically in Fig 3.6I) (p<0.05 for comparisons). The greatest reduction in wall thickness was observed using either the specific serine elastase or MMP inhibitors, 1K (64% fold decrease) and GM-6001 (45% reduction).

**Tenascin-C Antisense and Matrix Metalloproteinase Activity**

Since reduced TN was associated with regression of vessel wall thickness following MMP or elastase inhibition, we next addressed whether selective suppression of TN would reproduce these effects. The TN antisense expression vector was administered using the HVJ-liposome method, that facilitates a high rate of delivery to blood vessels *in vivo* (Yonemitsu, et al., 1996). To evaluate the relative efficiency of this methodology in our model, transfections using a CAT construct were performed. Relative to mock transfected controls (Fig 3.7A), abundant CAT immunostaining was observed throughout the vessel media, adventitia, and along the endothelium (Fig 3.7B).
TN, detected by immunohistochemistry, was present in PAs of MCT-treated rats and increased over the 8 days in organ culture with no significant difference between mock and sense transfected vessels (Fig 3.7C, densitometric quantification) (p<0.05). However, in PAs transfected with antisense cRNA, a significant reduction in TN deposition, to below pre-culture levels, was observed (56% relative to sense-transfected) (Fig 3.7C) (p<0.05). The reduction in protein was attributed to antisense/ribozyme mediated 58% loss of both the 7.3 and 6.4 kb TN mRNA transcripts compared to sense-transfected vessels and observed as early as 18 hours following transfection (Fig 3.7D) (p<0.05). TN modulation by antisense was confirmed to be downstream of MMP activity as no difference in MMP-2 activity was detected (data not shown).

Antisense Suppression of TN and Induction of Apoptosis

We next determined whether decreasing TN by antisense impairs cell survival and leads to apoptosis (Fig 3.8A). In freshly isolated MCT-treated PAs, apoptosis was not observed. In mock and sense-transfected vessels, 7-10% of cells were TUNEL positive after 8 days in organ culture, but in antisense transfected vessels, the level of apoptosis was increased to ~40% of cells (p<0.05). We confirmed that the TUNEL positive cells were, in fact, associated with nuclear condensation and fragmentation, and DNA laddering. PAs transfected with the sense construct displayed mostly normal nuclear staining and morphology assessed by propidium iodide labeling, whereas in antisense-transfected vessels. ~12% of nuclei exhibited bright focal centers, indicative of DNA chromatin condensation (Fig 3.8B) (p<0.05). Nuclear fragmentation was also observed in ~7-8% of cells in TN antisense-transfected vessels, whereas very few such cells were noted in sense control PAs (<2%) (Fig 3.8B) (p<0.05). DNA laddering, a hallmark of apoptosis, resulting from degradation of nucleosomal DNA was observed in sense organ cultures, but antisense transfection resulted in a 3 fold increase in intensity of these bands (Fig 3.8C) (p<0.05).
**TN Antisense, Vascular Cell Proliferation and Vascular Thickening**

To establish whether suppression of SMC proliferation following proteinase inhibition was also observed with TN antisense, PCNA immunohistochemistry was performed (Fig 3.9A). A significant induction in PCNA positivity was detected above pre-culture levels in both the mock and sense controls as well as with antisense-transfection (p<0.05 for each comparison). Thus loss of TN by antisense failed to suppress proliferation in PA organ culture. Since suppressing TN by antisense induced apoptosis but did not arrest proliferation, the net effect on wall thickness was assessed morphometrically (quantified in Fig 3.9B). While a significant increase in wall thickness, above day 21 controls, was observed in mock and sense transfected vessels (p<0.05), in antisense-transfected arteries, wall thickness was similar to that observed in freshly isolated, MCT-treated PAs (p<0.05).

**Tenascin-C Antisense and Osteopontin**

The presence of PCNA positive cells in the antisense-treated PAs, suggested incomplete transfection with antisense or proliferation in the absence of TN. Co-distribution of TN and PCNA on serial sections was present in sense vessels (Fig 3.10A, B & E). Conversely, while ~24% of all PCNA positive cells (Fig 3.10D) were associated with TN (Fig 3.10C) following antisense transfection, and likely represented untransfected cells, the remaining ~76% of cells were proliferating in a TN-independent manner (Fig 3.10E). Thus, antisense appeared to allow the expansion of a SMC subpopulation that is TN-negative.

TN amplifies the response to growth factors by ligating β3 integrin receptors, altering the cytoskeleton and causing clustering and enhancing activation of growth factor receptors. OPN, another ECM molecule, that contains an RGD site required to ligate β3 integrins, mediates vascular endothelial cell survival (Scatena, et al., 1998), and is produced by SMCs during disease (O'Brien, et al., 1994). We therefore investigated, by immunostaining,
whether OPN was present or upregulated. The diffuse, sometimes focal pattern of OPN deposition in sense-transfected PAs was reduced relative to pre-culture levels after 8 days in organ culture (Fig 3.11A & E). With suppression of TN by antisense, however, OPN immunoreactivity was markedly increased and localized primarily to intense, dense foci (Fig 3.11C & E) (p<0.05). In serial sections, there appeared to be an enhanced co-distribution of PCNA positive cells with intense OPN staining in antisense vessels (Fig 3.11). The suppression of TN and concomitant upregulation of OPN in antisense PAs were selective effects, as other ECM molecules such as fibronectin, elastin and collagen were not altered in proportion to wall thickness (Fig 3.11E).

Proteinase Inhibition and Osteopontin
Induction of OPN could account for the concomitant proliferation of SMCs resulting in arrest but not regression of PA hypertrophy, since we verified that similar to TN, OPN expression in tissue sections from inhibitor-treated cultures (Fig 3.12B, GM-6001; & D, 1K) was also reduced compared to vehicle-treated cultures (Fig 3.12A, DMSO; & C, PEG), as quantified in Fig 3.12E (p<0.05).

To further explore the role of OPN as a SMC survival factor, and to ensure uniform delivery of exogenous OPN, we used a previously characterized PA SMC culture model (Jones, et al., 1997b). We cultured rat PA SMCs with the proteinase inhibitor GM-6001, which suppresses TN (Jones, et al., 1997b), and showed induction of apoptosis (~50% cell loss over 48 hours, detected as DePsipher positivity described in Methods). This rapid assay for cultured cells avoids the fixation process necessary for tissue pieces. Addition of exogenous OPN (Fig. 3.13A), prevented the loss in cell number through the suppression of apoptosis (reduced by ~5 fold) (p<0.05).
**αvβ3 Integrin Blockade, Vascular Cell Apoptosis and Vascular Regression**

As TN or OPN-dependent survival has been shown to involve the ligation of αvβ3 integrins (Jones, et al., 1997b; Scatena, et al., 1998), we determined whether the absence of matrix interaction with this integrin could account for apoptosis and vascular regression. Hypertrophied pulmonary artery organ cultures were, therefore, treated with the functional blocking antibody, LM609 as described in the Methods. An apoptotic response was observed (~5.8 fold induction) similar to that detected with proteinase inhibitors (Fig. 3.13E. and quantified in F), while untreated vessels (Fig. 3.13C, after culture; & B, prior to culture) and IgG (Fig. 3.13D) controls maintained low levels of TUNEL positivity (p<0.05). This was again associated with regression of vascular hypertrophy (Fig. 3.13G) (p<0.05).
Figure 3.1

Tenascin-C Antisense cRNA. The hypothesized conformation of the TN antisense cRNA using the U1Bam pZeo mut Eco RI/Spe I expression vector backbone (a gift from Drs. H.C. Dietz and R.A. Montgomery, Johns Hopkins, Baltimore MD; schematic modified from that presented in Montgomery, R.A. and Dietz, H.C. Human Mol. Gen. 1997; 6:519-525). The EcoRI and SpeI restriction sites are underlined. Hairpin loops at the 5' and 3' ends are shown and the 32bp antisense RNA described in the text is constructed to incorporate a hammerhead ribozyme such that it folds and hybridizes to the target TN mRNA at the location of the GUC consensus cleavage site (arrow; site of cleavage).
Figure 3.2

Elastase and Matrix Metalloproteinase Activity. Elastase assays were used to detect net elastolytic activity in conditioned medium harvested from 8 day old PA cultures in the presence or absence of either the serine elastase inhibitors, α1-PI and 1K, active against endogenous vascular elastases or the MMP inhibitor, GM-6001 (A). Suppression of elastase activity was induced by all three inhibitors. Gelatin zymography, performed on tissue extracts of PAs grown for 8 days in media supplemented with either α1-PI or GM-6001, or with the administration vehicle controls for each inhibitor, enabled visualization of MMP activity (B). The most predominant bands ran as a doublet at a molecular weight of ~56 and 52 kD as shown in the zymogram, representative of three such experiments and summarized graphically. kD = kilodaltons, Bars = mean + SEM (n = 3), * = p<0.05 compared to administration vehicle controls.
Figure 3.3

Regulation of Tenascin-C by Proteinase Inhibitors. Representative photomicrographs of TN immunohistochemistry indicate that TN is increased at 21 days following MCT-injection (A), relative to PAs from saline-injected rats which show only faint staining along the outer media/adventitial border (B). TN continues to be abundantly deposited throughout the vessel wall in vehicle treated controls (C, PBS vehicle for $\alpha_1$-PI; E, DMSO vehicle for GM-6001; & G, PEG vehicle for 1K). In contrast, incubation with the proteinase inhibitors $\alpha_1$-PI (D), GM-6001 (F), and 1K (H) reduces TN. Bar = 25$\mu$m. Semi-quantitative analysis reflected these differences (not shown).
Figure 3.4

*Apoptosis in Hypertensive and Normotensive Pulmonary Arteries.* Apoptotic vascular cells were identified as bright green fluorescent nuclei following TUNEL labeling. Apoptosis was minimal prior to culture, 21 days after either MCT (A) or saline-injection (B), as only propidium iodide counterstaining can be observed. PAs cultured in media supplemented with the vehicles for the inhibitors did not show a change from pre-culture levels (C, PBS for α1-PI; E, DMSO for GM-6001; & G, PEG for 1K). However, in the presence of either α1-PI (D), GM-6001 (F), or 1K (H) a widespread induction of apoptosis occurred. Bar = 25μm.
Figure 3.5

Quantitative Analysis of Apoptosis and Proliferation. Counts of apoptotic cells, assessed by TUNEL positivity, are displayed. **A,** Proteinase inhibitors α1-PI, GM-6001, and 1K induce apoptosis in MCT vessels assessed following 8 days in organ culture. **B,** Induction of apoptosis with proteinase inhibitors is not observed in control PAs from saline-injected rats. **C,** Immunohistochemistry for PCNA was used as an indicator of proliferating vascular cells and quantified as a percent of total cells. Proliferation, which is induced by MCT is greatly increased after 8 days in organ culture in the presence of inhibitor vehicles (except DMSO) and suppressed by all three inhibitors. Bars = mean \( \pm \) SEM of \( n = 3 \) vessels (\( n = 6 \) for wall thickness graph), \(* = p<0.05\) compared to vehicle control, and \( \dagger = p<0.05\) compared to pre-culture MCT.
Figure 3.6

Proteinase Inhibitors and Regression of Vascular Hypertrophy. Representative movat pentachrome stained sections are shown in A-H. An increase in vessel thickness following MCT-injection (A), relative to that following saline-injection (B), but prior to placement into organ culture is shown. Vessels cultured in the absence of proteinase inhibition showed a further increase in wall thickness over 8 days, (C, PBS for α1-PI; E, DMSO for GM-6001; & G, PEG for 1K), whereas wall thickness was reduced to or below values of saline vessels when PAs were cultured with α1-PI (D), GM-6001 (F), or 1K (H). While the size of control, vehicle-treated vessels is such that they cannot be shown in entirety, differences in wall thickness are quantitatively displayed in I. Bar = 25 μm, graph bars = mean ± SEM of n = 6. * = p<0.05 compared to vehicle control, and † = p<0.05 compared to pre-culture MCT.
Figure 3.7

Distribution of Tenascin-C Antisense Transfection and Suppression of Tenascin-C. Distribution of plasmid DNA following transfection was assessed indirectly by immunostaining for chloramphenicol acetyltransferase transfection (CAT), a gene product from another plasmid but delivered by the same HVJ-liposome technique. HVJ-liposomes are effective in transfecting the cells throughout the vessel wall ex vivo at high efficiency. Relative to IgG stained controls (A), approximately 75-80% of cells are positive for CAT (B). Densitometric analysis of TN immunostaining (C) revealed an increase above pre-culture levels with mock or sense transfection. This was not only suppressed with TN antisense, but values were reduced below pre-culture levels. Reduction in TN protein assessed by immunohistochemistry correlated with decreased mRNA levels for the two TN transcripts as shown in the representative blot and densitometric data (D). With TN antisense, a decrease in TN mRNA transcripts at 7.3 and 6.4kb was observed when normalized for hybridization and loading by GAPDH hybridization. Bar = 25μm, graph bars = mean + SEM (n = 4), kb = kilobases, * = p<0.05 compared to sense and mock transfected, and † = p<0.05 compared to pre-culture MCT.
Figure 3.8

*Tenascin-C Antisense Transfection Induces Apoptosis.* In A, the number of apoptotic vascular cells under each condition reflect a slight induction in mock and sense-transfected PAs relative to PAs prior to culture. In antisense transfected PAs, however, a marked increase was observed above these levels. To assess the stringency of TUNEL assays, additional characteristics of apoptosis were examined. DNA of cells in antisense transfected tissue sections showed evidence of increased nuclear condensation and fragmentation quantified as a percent of total cells in (B). DNA laddering was visualized as ~180bp multimeric DNA fragments that were, by densitometry, ~3 fold more pronounced in antisense versus sense transfected PAs (C). Extracts from PAs prior to culture show no evidence of such multimers. Bars = mean + SEM of n = 4 vessels (n = 3 for laddering), kb = kilobases. * = p < 0.05 compared to sense and mock transfected and † = p < 0.05 compared to pre-culture MCT.
Figure 3.9

*Effect of Tenascin-C Antisense on Proliferation and Wall Thickness.*  
A, Quantification of differences in PCNA positivity indicated increased and similar levels in mock, sense and antisense treated PAs from MCT-injected rats in organ culture.  
B, Measurements of wall thickness indicate an increase in sense and mock vessels over 8 days in culture, while antisense treatment results in wall thicknesses statistically similar to freshly harvested PAs.  
Bars = mean + SEM of n = 6 vessels (n = 4 for PCNA graph), * = p<0.05 compared to sense and mock controls and † = p<0.05 compared to pre-culture MCT.
**Figure 3.10**

*A Tenascin-Independent Smooth Muscle Cell Population.* Suppression of TN via antisense results in the expansion of a proliferating, TN-independent SMC population. Representative photomicrographs of serial sections immunostained against either TN or PCNA are shown in A and B respectively, 8 days after sense transfection, and in C and D following antisense transfection. While all PCNA positive cells were associated with TN in sense-transfected vessels, loss of TN by antisense resulted in 76% of SMCs proliferating in the absence of TN, as displayed graphically in E. Bars = 25μm and graph bars = mean + SEM of 4 vessels.
**Figure 3.11**

*Tenascin-C Antisense Induces Upregulation of Osteopontin with Proliferating Smooth Muscle Cells.* The amount of OPN, an alternative αvβ3 ligand, deposited within the vessel wall was assessed. Representative photomicrographs illustrate that diffuse staining was observed in the sense control (A), (similar to pre-culture observations not shown). This was in contrast to abundant, dense focal deposition of OPN with TN antisense (C). The association between OPN and PCNA positivity was evident in antisense (C & D) relative to sense PAs (A & B). This increase in OPN was selectively exhibited in antisense cultures, as FN, elastin and collagen, normalized to changes in vessel thickness, were similar (quantified in E). Bars = 25μm, graph bars = mean + SEM of 4 vessels, and * = p<0.05 compared to sense control.
Figure 3.12

Suppression of Osteopontin by Proteinase Inhibition. Detection of OPN, by immunohistochemistry, in PAs cultured in the presence or absence of selective proteinase inhibitors. Representative photomicrographs indicate that, while OPN is deposited in the absence of proteinase inhibition (with vehicles: A, DMSO for GM-6001; & C, PEG for 1K), the presence of GM-6001 (B) or 1K (D) resulted in the loss of OPN immunoreactivity. As quantified in E, this retrospective examination showed that, like TN, OPN was suppressed by proteinase inhibition. Bars = 25μm, graph bars = mean + SEM of 4 vessels, and * = p<0.05 compared to vehicle control.
Figure 3.13

*Osteopontin and αvβ3 Mediated Smooth Muscle Cell Survival.*  A, Effect of exogenous OPN on apoptosis of rat PA SMCs following MMP inhibition associated with loss of the SMC survival ligand TN. The addition of recombinant OPN to collagen gels prevented loss of cell number and rescued the SMCs from apoptosis, assessed by loss of mitochondrial membrane potential, DePsipher positivity, as described in the Methods. Next we investigated the effect of αvβ3 integrin blockade in inducing apoptosis in hypertrophied PA organ cultures. Representative photomicrographs indicate that while apoptosis (bright green, TUNEL positive fluorescent nuclei) was minimal or absent in PAs after MCT-injection both prior to culture, day 21 (B), and after one week of culture either untreated (C) or treated with control IgG (D), unligation of the αvβ3 integrin with LM609 antibodies resulted in an abundant apoptotic response (E). This apoptosis, quantified in F, was associated with regression of vascular hypertrophy as evident in the photomicrographs, and as quantified in G. Bars = 25μm, graph bars = mean + SEM of 4 dishes or vessels, * = p<0.05 compared to DMSO control in A and IgG control in F and G, and † = p<0.05 compared to GM-6001 in A and pre-culture MCT (Day 0) in F and G.
DISCUSSION

PA organ culture was used to show that inhibition of elastases and MMPs suppresses TN, and this not only arrests progression but leads to regression of the thickened vessel wall in association with apoptosis and reduction in ECM components. An antisense technique effectively suppressed TN and caused a similar degree of SMC apoptosis, but proliferation of a group of TN-negative SMCs continued unaffected in association with enhanced deposition of an alternative ECM protein survival factor OPN. Indeed, exogenous OPN can rescue cultured SMCs from MMP-induced apoptosis associated with TN downregulation. Correspondingly, unligation of SMCs to TN and OPN, via blocking the $\alpha_v\beta_3$ receptor, induced apoptosis and regression, similar to proteinase inhibition. Taken together, this suggests that a PA SMC subpopulation is highly adaptive to survival ligand usage, stimulated by the growth-promoting proteolytic environment. Thus a strategy aimed either at inhibiting the general sequelae of proteolytic events, e.g., stimulation of ECM deposition and release of growth factors, or common survival signaling pathways may be more effective in inducing regression of disease than the more selective approach of targeting a specific ECM molecule.

In this study we adapted an organ culture system previously used to show the development of a neointima in porcine aorta in association with SMC proliferation (Koo and Gotlieb, 1991). Those studies showed that the mechanism of SMC proliferation is likely related to serine elastase activity (Oho, et al., 1995). Serine elastases are known to stimulate SMC proliferation by releasing growth factors such as FGF-2 from ECM proteoglycans (Thompson and Rabinovitch, 1996). Through the proteolysis of collagen, elastases can expose RGD binding sites (Kafienan, et al., 1998) which induce $\beta_3$ integrin-dependent upregulation of the matrix glycoprotein TN (Jones, et al., 1999), amplifying the SMC proliferative response by facilitating phosphorylation of growth factor receptors upon their ligation (Jones, et al., 1997b). In previous cell culture studies, stress unloading inhibited
MMPs, suppressed TN and induced SMC apoptosis. Recently, we showed that the response of SMCs, in hypertrophied but not normal arteries, to stress unloading in organ culture was similar (Cowan, et al., 1999). We, also, documented reduced elastase activity and showed that these effects translated into regression of the thickened vessel wall. It would be interesting to use available transgenic knockout mice to further explore the selective roles of MMPs, TN and OPN. The effects observed are, however, limited to hypertrophied vessels, and MCT does not induce pulmonary vascular disease in mice.

Increased activity of both serine elastases and MMPs is associated with vascular remodeling (Zempo, et al., 1994; Patel, et al., 1996; Thompson, et al., 1998; Wigle, et al., 1998) and using either serine elastase inhibitors or MMP inhibitors over the 8 days in organ culture resulted in a similar reduction in elastase and MMP activity. It would, therefore, appear that progressive medial hypertrophy depends on an interaction between MMPs and serine elastases. Serine elastases could allow for activation of MMPs directly or via inactivation of inhibitors or vice versa. For example, if the endogenous serine elastase is similar to other elastases such as neutrophil or pancreatic elastase, it could activate latent MMPs (Okada and Nakanishi, 1989), and degrade tissue inhibitors of matrix metalloproteinases (TIMPs) and, by destabilizing TIMP-MMP complexes, facilitate release of the active enzyme (Itoh and Nagase, 1995). Elastases also release elastin or fibronectin derived peptides as well as ECM-incorporated FGF-2 (Thompson and Rabinovitch, 1996) all of which can increase MMP expression and activity (Werb, et al., 1989; Landeau, et al., 1994; Miyake, et al., 1997). While elastases do not require proteolysis for activation, MMP-mediated degradation of native elastase inhibitors, such as \( \alpha_1 \)-PI (Mast, et al., 1991), may enhance vascular elastolytic activity. In addition, MMPs activated in association with endothelial injury may degrade matrix components leading to the loss of the basement membrane barrier (Emonard and Hornebeck, 1997), and allow for serum factors to stimulate SMC elastase activity (Thompson, et al., 1998; Wigle, et al., 1998). Alternatively, both serine
elastase and MMP activities are independently associated with progressive disease and inhibition of either serine elastase or MMP activity is sufficient to induce regression. Our previous studies examining MCT-induced PA hypertrophy, attributed the elevated elastase activity to a 20kD serine elastase (Zhu, et al., 1994). Matrix metalloelastases, however, were not evident, and non-elastolytic activity of MMPs was not assessed (Todorovich-Hunter, et al., 1992).

Evidence for the importance of a TN-SMC interaction in vascular biology comes from characterization of TN's tight developmental regulation and pathological re-expression (Hedin, et al., 1991; Jones and Rabinovitch, 1996; Kostianovsky, et al., 1997). Cell culture studies have identified a number of factors that influence TN expression (Chiquet-Ehrismann, et al., 1994; Rettig, et al., 1994), and these include MMPs that are also co-induced during vascular disease (Hedin, et al., 1991; Zempo, et al., 1994; Jones and Rabinovitch, 1996; Patel, et al., 1996). The mechanism involves proteolysis of native type I collagen and ligation of newly exposed RGD sites by β3 integrin receptors on SMCs (Jones, et al., 1997b). The subsequent intracellular signaling leads to transactivation of the TN promoter (Jones, et al., 1999). Furthermore, elastases can release stored matrix bound growths factors, such as FGF-2 (Thompson and Rabinovitch, 1996), which can also stimulate production of TN (Rettig, et al., 1994). How inhibition of elastases, also shown recently in the intact animal (unpublished data), or MMPs (Jones, et al., 1997b), reduce TN expression is not known, but this may be related to suppression of the β3 integrin-directed morphological changes and downstream signaling events previously described (Jones, et al., 1999).

The mechanism whereby suppression of TN, either resulting from reduced elastase or MMP activity or TN mRNA antisense, leads to SMC apoptosis is not known. Since, αvβ3 ligation to TN results in a re-organization of the SMC filamentous actin cytoskeleton, focal
adhesion morphology, and ultimately cell shape (Jones, et al., 1997b) regulating cell behavior (Folkman and Moscona, 1978; Chen, et al., 1997), loss of this ligation to TN may initiate the apoptotic cascade. Correspondingly, apoptosing cells can be rescued by addition of exogenous TN (Jones, et al., 1997b). Our studies documenting induction of SMC apoptosis with an αvβ3 functional blocking antibody would support suppression of αvβ3 ligation as the mechanism.

We further suggest that if SMCs respond similarly to endothelial cells, αvβ3 ligation may involve nuclear factor (NF)-κB related signaling (Scatena, et al., 1998). This would lead to transcriptional activation of inhibitor of apoptosis protein (IAP)-1 (Erl, et al., 1999), together with sustained ERK activity (Eliceiri, et al., 1998) and suppression of p53 and the cyclin-dependent kinase inhibitor p21WAF1/CIP1 (Stromblad, et al., 1996). Similar to our studies with cultured SMCs, blocking endothelial cell αvβ3 integrin interactions, with either cyclic peptides or functional blocking antibodies, induces apoptosis leading to vessel loss (Brooks, et al., 1994b; Choi, et al., 1994). Moreover, an αvβ3 integrin survival signal within the vasculature has been further supported by αv knockout mice which exhibit an embryonically lethal phenotype (Bader, et al., 1998). While evidence for an αvβ3-TN SMC survival pathway is strong, as provided by this report and others (Jones, et al., 1997b), these studies appear, at first, inconsistent with the presence of only a mild phenotype when TN is knocked out (Saga, et al., 1992). Reconciliation of these facts is likely to involve the presence of redundant cell survival signals, as our present data suggests, such as co-ordinate upregulation of OPN, an alternative αvβ3 integrin ligand.

Osteopontin is expressed by SMCs present in vascular disease (O'Brien, et al., 1994), and its role as a survival factor has been reported in endothelial cells (Scatena, et al., 1998). We have now shown that exogenous OPN can also support survival of SMCs when proteinase inhibition suppresses ECM survival ligands. Indeed, failure to expand a proliferative, TN-
negative subpopulation following proteinase inhibition correlated with loss of both TN and OPN. The possibility exists that OPN expression by SMCs is enhanced by proteinase activity enabling them to respond to pro-proliferative cues. While we have not identified other characteristics that differentiate the TN-dependent SMCs that apoptose following TN antisense treatment from the TN-independent cells that produce increased OPN, these results are in keeping with recent work by Stenmark and colleagues (Frid, et al., 1997) documenting heterogeneity of PA SMCs. Such heterogeneous populations suggest the potential for an adaptive response to alterations in cell matrix interactions in disease.

In our study, regression of hypertrophy involved both a loss of cellular and matrix components, while previous studies have addressed only a requirement for a reduction in cellularity (Pollman, et al., 1998). Loss of ECM, paradoxically in the presence of proteinase inhibitors, may have resulted from phagocytosis and intracellular degradation by vascular cells (Sawada and Inoue, 1997). Matrix degradation within vesicular membranes would have been inaccessible to the inhibitors used in our study. Alternatively, proteolysis of ECM may have been the effect of enzymes not targeted by the inhibitors used in our study, e.g., by release into the microenvironment (Ormerod, et al., 1993), of upregulated apoptosis associated enzymes with ECM degrading capacities.

These organ culture studies have suggested a novel strategy to induce regression of vascular disease. Proteinase inhibitors produce a robust apoptotic response accompanied by loss of ECM, whereas the heterogeneity and plasticity of vascular SMC subpopulations appear to limit the usefulness of targeting specific ECM survival factors.
CHAPTER FOUR

Loss of β3 Integrin Ligation to Tenascin-C Induces Vascular Smooth Muscle Cell Apoptosis
ACKNOWLEDGEMENTS

A significant contribution to this work was made by a summer student, Catherine Lam, whose dedication to the project was gratefully appreciated. Catherine was responsible for carrying out many of the experiments described in the following pages.

INTRODUCTION

The hallmarks of lesion development, in progressive pulmonary vascular disease, are similar to those observed in other occlusive vascular diseases (Ross, 1986; Billingham, 1992), and include smooth muscle cell (SMC) proliferation and the abnormal neosynthesis of extracellular matrix (ECM) components, like elastin, collagen, fibronectin and tenascin-C (Prosser. et al., 1989; Jones. et al., 1997a; Rabinovitch, 1998). In a normal vessel, SMCs are maintained in a quiescent contractile phenotype by signals through cell surface receptors including integrins (Thyberg, 1996; Thyberg, 1998) but, in response to endothelial cell injury, ECM-degrading proteinases are upregulated and this perturbs both cell-cell and cell-matrix interactions. Ligation to this altered extracellular matrix is associated with SMC phenotypic changes which we have addressed in previous cell culture studies (Jones and Rabinovitch, 1996; Jones. et al., 1997b; Jones, et al., 1999) and in previous chapters in this thesis in organ culture.

Tenascin-C (TN) is a large extracellular matrix glycoprotein that is expressed in actively remodeling tissue in both embryonic development and adult life (Crossin, 1994; Mackie, 1997). Cell- and tissue-specific expression of TN is regulated by a variety of factors (Rettig. et al., 1994; Chiquet-Ehrismann, 1995) including β3 integrin signaling upon SMC ligation to cryptic RGD (arginine-glycine-aspartic acid) sites revealed in matrix metalloproteinase (MMP) -degraded collagen (Davis, 1992; Sriramarao, et al., 1993; Jones, et al., 1999). Signaling through β3 integrins induces TN expression (Jones, et al., 1999) and promotes heightened growth factor responsiveness (Jones and Rabinovitch, 1996). In
fact, the proteolytic release of growth factors from ECM is coordinated with proteinase-dependent TN-mediated signaling through growth factor receptors (Thompson and Rabinovitch, 1996). While the cellular mechanism for increased proliferation in the presence of TN has been documented (Jones, et al., 1997b), the process leading to apoptosis following TN withdrawal has not been examined.

Increasing evidence supports a role for adhesion to ECM in signaling required for survival of many cell types. For example, a restructuring of three-dimensional tissue organization, as observed during mammary involution, results in withdrawal from the cell cycle and apoptosis of mammary epithelial cells (Boudreau, et al., 1996), as does upregulation of stromelysin-1 activity or disruption of β1 integrin ligation to ECM (Boudreau, et al., 1995). Similarly, deprivation of ECM ligation resulted in apoptosis of endothelial cells that was related to integrin-mediated (Meredith, et al., 1993) cell shape changes (Re, et al., 1994), which have been shown to be an important determinant of cellular function (Folkman and Moscona, 1978; Chen, et al., 1997). The requirement for cell-ECM adhesive interactions in the regulation of cell survival is likely vital for successful morphogenesis and to ensure the maintenance of tissue architecture, with the demise of cells that detach from their resident location or attach at an inappropriate site (Ruoslahti, 1997; Giancotti and Ruoslahti, 1999). Indeed, during physiological processes requiring cellular migration and tissue invasion, cell-matrix survival signals are mediated by expression and occupancy of a separate repertoire of integrins, such as observed with the αvβ3 integrin on endothelial cells during angiogenesis (Brooks, et al., 1994a; Drake, et al., 1995; Boudreau, et al., 1997). Thus, as αvβ3 ligation to TN results in re-organization of the SMC filamentous actin cytoskeleton, focal adhesion morphology and ultimately cell shape (Jones, et al., 1997b), loss of this ligation to TN may initiate the apoptotic cascade.
The studies described in the preceding chapters of this thesis, using a hypertensive pulmonary artery organ culture model, have supported a role for αvβ3 integrin ligation in survival of SMCs within the intact vessel wall (Cowan, et al., 1999; Cowan, et al., 2000). Specifically, as Stenmark and coworkers (Frid, et al., 1994; Wohrley, et al., 1995; Frid, et al., 1997; Dempsey, et al., 1998) have reported that native SMC populations have considerable heterogeneity, we have shown that this heterogeneity extends to survival ligand usage. That is, SMCs can survive if expression of either TN or OPN is induced, as they are both αvβ3 integrin ligands. Conversely, αvβ3 integrin blockade effectively compromises survival to initiate regression of a thickened vessel wall (Cowan, et al., 2000).

In this study, we further investigated the relationship between β3 integrin signaling and SMC survival. First, we confirm that the induction of PA SMC apoptosis in association with suppression of TN and OPN, in response to MMP inhibition or floating collagen gels, can be recapitulated using a rat aortic SMC line (A10 cells). Next, we show that, similar to organ culture, SMC survival was dependent upon β3 integrin ligation, as the specific blockade of this integrin subunit in the presence of TN resulted in apoptosis of SMCs. Examining the localization of β3 integrins under apoptotic conditions revealed a diffuse distribution, in contrast to non-apoptotic conditions, in which these integrins were found in focal adhesion-like formations at the cell periphery. Finally, reclustering of β3 integrins rescued the SMCs from apoptosis despite the absence of matrix survival ligands, suggesting a functional paradigm whereby extracellular matrix ligand interactions with β3 integrins promote SMC survival.
MATERIALS AND METHODS

A10 Smooth Muscle Cell Culture

A10 vascular smooth muscle cells, a fetal rat thoracic aorta cell line (American Tissue Culture Collection, VA), were routinely maintained in Medium 199 (M199) containing 10% fetal bovine serum (FBS) (Intergen, Purchase, NY), 10 unit/ml penicillin G sodium, 10mg/ml streptomycin sulfate, 0.25 mg/ml amphotericin B and 0.1 mg/ml gentamicin sulfate (Gibco-BRL). Cells were passaged by trypsinization using 0.05% trypsin/EDTA (Gibco-BRL). Type I collagen gels were prepared according to the manufacturer's recommendation (Vitrogen 100, Collagen Corp., Fremont, CA). Briefly, vitrogen 100 collagen was mixed on ice with 10X PBS, containing 0.005 mg/ml phenol red, and 0.1 mol/L NaCl at a ratio of 8:1:1 for a final collagen concentration of 2.48 mg/ml and adjusted to pH 7.4. The neutralized collagen solution was then added to ice-cooled culture and dispersed using a cell spreader. Fibrillogenesis was initiated overnight in a humid 5% CO₂ environment at 37°C. Before plating cells, collagen substrates were rinsed extensively (3x3 hours) with serum-free M199. Cells were plated at a density of 1.66x10⁴ cells/cm² on collagen in M199 supplemented with 2% FBS. Twenty-four hours after plating, cells were rinsed three times with serum-free medium, and then with M199 plus 0.1% bovine serum albumin (BSA) (Boehringer-Mannheim; Fraction V) and 0.1% FBS. Cultures were then maintained for an additional 48 hours in M199 with 0.1% BSA/FBS and supplemented with 50 ng/ml of epidermal growth factor (EGF).

Tenascin-C Suppression

Suppression of the vascular SMC survival factor tenascin-C was performed as previously reported (Jones, et al., 1997b). Briefly, TN expression became inapparent by western immunoblot when cells were incubated with MMP inhibitors or were cultured on gels which were 'floated'. For MMP inhibition studies, 2μm of GM-6001 (a kind gift from Ligand Pharmaceuticals, San Diego, CA) in dimethylsulfoxide (DMSO), or an equivalent volume of
DMSO alone, were added to cultures 24 hours after plating. GM-6001, an N-[2(R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophane methylamide, is a non-cytotoxic, synthetic inhibitor that functionally and specifically inhibits MMP activity, by complexing with the zinc atom found in the active site of MMPs as well as by interfering with substrate interaction. Floating of collagen gels was performed by detaching the collagen from the plastic substratum with the use of a spatula.

Detection of Apoptosis

As an indicator of apoptosis, loss of mitochondrial membrane potential was monitored with the DePsipher detection system (R&D Systems, Minneapolis, MN). DePsipher is a lipophilic cation (5, 5', 6, 6', 7 tetrachloro-1', 1', 3, 3'-tetraethylbenzimidazolyl carbocyaninIodide) which aggregates upon membrane polarization forming an orange-red fluorescent compound. Disruption of membrane potential prevents access of the dye to the transmembrane space and thus it remains or reverts to its green monomeric form. SMCs were stained in the living state by a 15 minute incubation with the DePsipher dye in stabilizing buffer followed by washing and visualization by fluorescent microscopy. DePsipher positive cells correlated with evidence of DNA fragmentation, a hallmark of apoptosis. Briefly, to extract SMCs adherent to the collagen substrates, gels were digested with 1 mg/ml collagenase type II (Sigma Chemical Co.) for 1 hour at 37°C. Liberated cells were pelleted by centrifugation at 4°C for 10 min at 300 g and resuspended in Hanks Buffered Saline Solution (HBSS) containing 0.6% NP-40, 1mg/ml RNAsse and 0.1 mg/ml propidium iodide. After a 30 minute incubation, cells were analyzed by flow cytometry (Excalibur FACS, Beckman Coulter Inc., Fullerton, CA) and SMCs exhibiting fluorescence below the normal diploid DNA content, gated as ‘M1’, were considered apoptotic.
Cell Counts

The number of cells on collagen gels was determined 72 hours after plating. The cell number was determined by counting aliquots using an improved Neubauer hemocytometer (American Optical, Buffalo, NY). Three hours following seeding of cells onto the gels, attachment efficiencies were determined by counting the number of cells in the medium. The number of attached cells (number of cells seeded - number of cells in the medium) was then compared to the number of smooth muscle cells that could be extracted from collagen gels after treatment and resuspended in phosphate buffered saline (PBS) containing 0.05% trypsin for counting, as described above. The ratio was then expressed as a percentage.

The number of cells retained on collagen gels was determined 72 hours after plating. Three hours following seeding of cells onto the gels, attachment efficiencies were determined by counting the number of cells in the medium using an improved Neubauer hemocytometer (American Optical, Buffalo, NY). The number of attached cells (number of cells seeded - number of cells in the medium) was expressed as a percentage of the number of cells seeded. Smooth muscle cells were extracted from collagen gels as described above and resuspended in phosphate buffered saline (PBS) containing 0.05% trypsin. The cell number was determined by counting aliquots using a haemocytometer.

β3 Integrin Blocking Studies

Smooth muscle cells were pre-incubated, for 1 hour on ice, and cultured in media supplemented with either 15μg/ml of an anti-αvβ3 functional blocking antibody, LM609 (Chemicon, Temecula, CA), 25 μg/ml of an anti-rat β3 integrin monoclonal antibody (CD61, Pharmingen), or 15μg/ml of control IgG (PharMingen, San Diego, CA), as previously described for cell culture (Jones, et al., 1997b).
**Immunofluorescent Detection of β3 Integrins**

The localization of β3 integrins on SMCs was assessed by immunofluorescence. Accordingly, after culturing with either GM-6001 or DMSO, SMCs were rinsed in PBS and fixed in acetone at -20°C for 1 minute. Cells were rehydrated in PBS, blocked in PBS containing 1% BSA and 10% goat serum for 1 hour at 37°C, and incubated with a fluorescein-conjugated goat anti-mouse antibody diluted 1:100 in PBS/1% BSA for 30 minutes at 37°C, before washing and visualization by epifluorescence.

**β3 Integrin Cross-Linking Studies**

To cluster β3 integrins, SMCs cultured on collagen gels were incubated with 150 μg/ml of an anti-β3 monoclonal antibody diluted in M199/0.1% BSA as previously reported (Jones, et al., 1997b). The medium was then replaced with fresh medium containing either 20 μg/ml of goat anti-mouse IgG F(ab')2 fragment (ICN Biomedicals, Inc.) and GM-6001 or with GM-6001 alone. Additional control cultures were incubated with 15μg/ml of control IgG (PharMingen, San Diego, CA) followed by medium containing GM-6001 in the presence or absence of the IgG F(ab)2 fragments, or cultured in the absence of a pre-incubation with IgG F(ab')2 fragments and GM-6001, or with GM-6001 alone or an equal volume of DMSO.
RESULTS

Matrix metalloproteinase suppression, through floating of collagen gels or targeted inhibition, induces apoptosis of smooth muscle cells

As MMPs regulate TN deposition in A10 SMC culture (Jones, et al., 1999), we determined whether A10 cells will apoptose following direct or indirect inhibition of MMPs, associated with TN withdrawal. Loss of mitochondrial membrane potential was used as a cell culture indicator of apoptosis, in which red mitochondrial fluorescence indicates normal membrane potential and cell viability, and green fluorescence indicates loss of a mitochondrial membrane potential as observed during apoptosis. This technique revealed that while some apoptotic A10 SMCs were detected in control cultures (attached with or without DMSO, the vehicle for GM-6001; Figure 4.1: photomicrograph ‘DMSO’ and graph), MMP inhibition either directly, with GM-6001 (Figure 4.1: photomicrograph ‘GM-6001’) or, indirectly, associated with floating collagen gels, induced a ~2.5 fold induction in apoptosis (Figure 4.1: graph) (p<0.05). These results and the detection of apoptosis by this method correlated with changes in cell number and the presence of DNA fragments as monitored with propidium iodide flow cytometry. Hypodiploid DNA fragments, gated as ‘M1’ on flow cytometry plots, were increased ~3.1 fold in MMP inhibited cultures (Figure 4.2: plot ‘GM-6001’ and graph A) compared to controls (Figure 4.2: plot ‘DMSO’ and graph A) (p<0.05). This induction of apoptosis was associated with an ~61% loss in cell number (Figure 4.2: graph B). Thus, this culture system represents a model by which to study MMP suppression-induced smooth muscle cell apoptosis.

β3 integrin blockade recapitulates the apoptotic response

Since SMC ligation to TN involves αvβ3 integrins (Jones, et al., 1997b), we performed β3 integrin blocking studies to determine whether β3 ligation is critical to survival. A similar background level of apoptosis, assessed by DePsipher fluorescence, was detected following incubation with non-specific IgG antibodies (Figure 4.3: photomicrograph ‘IgG’).
However, the addition of either an αvβ3 functional blocking antibody, LM609 (Figure 4.3: photomicrograph ‘LM609’), or a β3 antibody alone (Figure 4.3: photomicrograph ‘CD61’) induced an ~2.3 fold induction of apoptosis (Figure 4.3: graph A) (p<0.05). These results correlated with a ~53% loss of A10 SMCs in LM609 or CD61 treated cultures as compared to IgG controls (Figure 4.3: graph B). This confirms that β3 ligation is required for smooth muscle cell survival.

**Induction of apoptosis is associated with unclustering of β3 integrins**

We next addressed whether loss of TN ligation during apoptosis is associated with a redistribution of β3 integrins. Changes in β3 integrin localization on A10 SMCs were examined following induction of apoptosis by MMP inhibition with GM-6001. Immunolocalization of β3 integrins indicated clustering in focal adhesion contact-like formations on the cell periphery in vehicle control (DMSO alone) samples (Figure 4.4: ‘DMSO’ photomicrographs). This distribution was altered following treatment with GM-6001 in which diffuse, barely evident staining was observed (Figure 4.4: ‘GM-6001’ photomicrographs). This study links unclustering of β3 integrins with the induction of smooth muscle cell apoptosis.

**Smooth muscle cell survival is enabled by β3 integrin clustering**

Next, we evaluated the ability of β3 integrin clustering to suppress SMC apoptosis. Matrix metalloproteinase inhibitor treated cultures were further supplemented with either non-specific control antibodies, IgG (Figure 4.5: photomicrograph ‘GM+IgG’), β3 integrin antibodies, CD61 (Figure 4.5: photomicrograph ‘GM+CD61’), or with IgG and F(ab’)2 antibody fragments directed to the antibody Fc domain (Figure 4.5: photomicrograph ‘GM+IgG+F(ab’)2) and all exhibited a similar level of apoptosis (Figure 4.5: graph). When provided with both CD61 and F(ab’)2 fragments (Figure 4.5: photomicrograph ‘GM+CD61+F(ab’)2), SMC apoptosis was reduced ~43% becoming similar to untreated
cultures (Figure 4.5: graph) \((p<0.05)\). Thus, 83 clustering rescues smooth muscle cells from apoptosis.
Figure 4.1

Representative photomicrographs of A10 smooth muscle cells cultured on collagen gels and stained for changes in mitochondrial membrane potential with the DePsipher fluorescent dye. Green fluorescence indicates loss of mitochondrial membrane potential, as predominantly observed in smooth muscle cells treated with the matrix metalloproteinase inhibitor, GM-6001, or alternatively, a red fluorescence, predominantly observed in cells cultured with the control vehicle, DMSO, indicates a normal mitochondrial membrane potential. Differences in DePsipher fluorescence, indicative of apoptosis, were quantified and graphed in the lower half of the figure together with results obtained by culturing smooth muscle cells on attached versus floating collagen gels. Original magnification = 400x; graph bars = mean + SEM of 4 dishes; and * = p<0.05 compared to controls (DMSO and attached).
Figure 4.2

Propidium iodide flow cytometry plots are presented in the upper half of this figure. DMSO, vehicle control condition, indicates that the A10 smooth muscle cell population is primarily in the G1/G0 phase of the cell cycle, gated as ‘M2’, a subpopulation are cycling in S/G2 phase, gated as ‘M3’, and some cells have hypodiploid DNA, gated as ‘M1’ and indicative of apoptotic cells. In contrast, while the majority of the flow cytometry profile is the same with GM-6001, (MMP inhibition), an expansion of the ‘M1’ apoptotic population is observed. Repeated studies for both matrix metalloproteinase inhibitor treatment and attached versus floating conditions are summarized graphically in A and correlated with cell counts shown in B. FL2-A indicates fluorescence; graph bars = mean + SEM of 4 dishes; and * = p<0.05 compared to controls (DMSO and attached).
Figure 4.3

The effect of β3 integrin blockade on A10 smooth muscle cell survival is shown by representative photomicrographs of DePsipher fluorescence. A10 smooth muscle cells cultured on collagen gels were presented with either an αvβ3 functional blocking antibody, LM609, a β3 antibody, CD61, or a non-specific control antibody, IgG. Differences in the level of apoptosis and overall cell number were quantified and plotted in graph A and B, respectively. Original magnification = 400x; graph bars = mean + SEM of 4 dishes; and * = p<0.05 compared to control IgG.
Figure 4.4

Changes in the localization of β3 integrins with apoptosis induction were monitored. Representative photomicrographs indicate that under non-apoptotic conditions, with the vehicle for the matrix metalloproteinase inhibitor DMSO, β3 integrins are localized to focal-adhesion contact-like formations, while under apoptotic conditions, with the matrix metalloproteinase inhibitor GM-6001, unclustering and loss from these formations is observed. Two examples are provided for each condition (upper and lower photomicrographs) at an original magnification of 1000x.
Figure 4.5

The contribution of β3 integrin clustering to A10 smooth muscle cell survival was addressed. Representative photomicrographs of DePsipher stained cells indicate that either the non-specific antibody, IgG (GM+IgG), the β3 antibody, CD61 (GM+CD61), or a mix of both IgG and Fc directed F(ab')2 antibody fragments (GM+IgG+F(ab')) do not prevent matrix metalloproteinase inhibitor (GM-6001) induced apoptosis. Alternatively, the artificial clustering of β3 integrins with a combination of CD61 and the F(ab')2 antibody fragments (GM+CD61+F(ab')) resulted in A10 smooth muscle cell survival despite matrix metalloproteinase inhibition. Differences in the presence of apoptosis were quantified and graphed in the lower part of this figure. Original magnification = 400x; graph bars = mean + SEM of 4 dishes; and * = p<0.05 compared to controls (IgG, CD61, and IgG+F(ab')2).
DISCUSSION
While numerous studies have correlated tenascin-C deposition with the presence of phenotypically altered proliferating SMCs in various pathological states (Hedin, et al., 1991; Majesky, 1994; Hahn, et al., 1995; Chen, et al., 1996; Jones and Rabinovitch, 1996; Jones, et al., 1997a; Wallner, et al., 1999b), only very recently has a role for TN been implicated in sustaining cell survival (Jones, et al., 1997b). In the present study, we have shown that TN-dependent survival is transduced through β3 integrins. We have, also, documented that loss of ligation to TN involves a dissociation of clustered β3 integrins on the cell surface and that the reclustering of these integrins, despite lack of TN, supports survival of SMCs.

Upregulation of survival ligands and their receptors have been reported in systemic vascular disease (Hedin, et al., 1991; Majesky, 1994; Hahn, et al., 1995; Chen, et al., 1996; Veinot, et al., 1999; Wallner, et al., 1999a; Wallner, et al., 1999b). In our studies systemic arterial SMCs derived from rat thoracic aorta (A10), were similar to primary cultured rat pulmonary artery SMCs, in that apoptosis was induced following MMP inhibition either directly or by using floating collagen gels (Jones, et al., 1997b). In addition, as in pulmonary artery organ culture (Cowan, et al., 2000), blockade of β3 integrins in A10 cells was effective in inducing apoptosis. Induction of endothelial cell apoptosis and vessel loss with the LM609 αvβ3 functional blocking antibody has also been used by Brooks and coworkers (Brooks, et al., 1994a; Brooks, et al., 1994b; Brooks, et al., 1995) to reverse tumour progression. Other groups have reported the ability to limit and arrest neointimal formation, following systemic vascular injury, by αvβ3 integrin blockade with antibodies or cyclic RGD peptides (Choi, et al., 1994; Srivatsa, et al., 1997; van der Zee, et al., 1998), but apoptosis was not assessed. Pollman et al (Pollman, et al., 1998) have, however, reported success in regressing systemic vascular lesions by inducing SMC apoptosis, albeit through bcl-x antisense.
It is well established that the extracellular matrix profoundly influences cellular function (Rana, et al., 1994; Zhu and Assoian, 1995; Boudreau, et al., 1996; Dike and Ingber, 1996; Chen, et al., 1997; Dike, et al., 1999) and that the receptors that transduce ECM-derived information participate in the determination of cell fate (Meredith, et al., 1993; Frisch and Francis, 1994; Boudreau, et al., 1995; Stromblad, et al., 1996). While we have previously shown that clustering of β3 integrins promotes cell proliferation through actin cytoskeletal reorganization, focal contact formation and concomitant growth factor receptor clustering (Jones, et al., 1997b), these studies now show that the clustering of β3 integrins by ECM survival factors also prevents vascular SMC apoptosis. The mechanism may also be related to the induction of focal adhesion contacts and the initiation of an intracellular signaling survival pathway. While integrins do not have any intrinsic kinase activity (Burridge and Chrzanowska-Wodnicka, 1996), their ligation to TN was associated with the assembly of focal adhesions and with the aggregation of numerous tyrosine phosphorylated proteins including focal adhesion kinase (FAK) (Hanks, et al., 1992; Schaller, et al., 1992; Schlaepfer, et al., 1994). A survey of SMC tyrosine phosphorylated proteins following ligation to TN identified an increase in a 125 kDa band attributed to FAK (Jones, et al., 1997b). Consistent with this, Frisch et al (Frisch, et al., 1996) showed that the transfection of a constitutively active FAK leads to transformation, adhesion-independent growth and suppression of apoptosis in epithelial cells. Conversely, Huggerford (Hungerford, et al., 1996) showed that competitive inhibition of FAK binding to the cytoplasmic domain of the β1 integrin subunit induced apoptosis in fibroblasts. Active FAK may mediate survival through a pathway involving the sequential phosphorylation of phosphoinositide 3-OH kinase (PI 3-kinase) and protein kinase B/Akt (PKB/Akt), as demonstrated by Khwaja (Khwaja, et al., 1997) in a study where inhibition of either kinase abrogated survival in epithelial cells. The cell-intrinsic death machinery targets of PKB/Akt include apoptosis promoting factors like BAD and caspase-9 and phosphorylation of these proteins by PKB/Akt results in loss-of-function (Datta, et al., 1997; Cardone, et al., 1998). In addition,
FAK regulates p53-mediated apoptosis, through protein kinase A and phospholipase A₂ (Illic, et al., 1998) and inactivation of p53 prevents apoptosis in FAK deficient cells.

Without implicating FAK directly, other groups have reported integrin adhesion-mediated suppression of apoptosis. Zhang et al (Zhang, et al., 1995) have shown the expression of the anti-apoptotic protein bcl-2 in response to Chinese hamster ovary (CHO) cell adhesion to fibronectin and downstream signaling associated with the cytoplasmic domain of the β₁ subunit of the α5β₁ integrin receptor. Also, an increased bcl-2/bax ratio has been observed in response to endothelial cell ligation to the αvβ₃ integrin as well as suppression of p53 and activation of the transcription factor nuclear factor (NF) -κB (Stromblad, et al., 1996; Scatena, et al., 1998). Very recently, NF-κB has been shown by Erl (Erl, et al., 1999) to upregulate expression of inhibitor of apoptosis protein (IAP) -1, which reportedly binds to and inactivates members of the caspase family of proteinases (Deveraux, et al., 1997; Roy, et al., 1997; Deveraux, et al., 1998). Thus, the clustering of β₃ integrins in the present study may activate one or more of these anti-apoptotic mechanisms, such that, the loss of signaling following unligation fails to prevent apoptosis.

As focal contacts are formed by SMC adhesion to TN (Jones, et al., 1997b) and are a pre-requisite for SMCs to respond to EGF (Jones and Rabinovitch, 1996), loss of ligation to TN in the presence of growth factors like EGF may also provide conflicting cell cycle signals thereby inducing SMC apoptosis, as has been suggested for mammary epithelial cells during involution (Boudreau, et al., 1996). These studies reinforce the concept that cell survival requires a co-ordination of integrin and growth factor signaling. Consistent with this, SMC ligation to TN via αvβ₃ integrins caused a redistribution of filamentous actin and co-clustered the αvβ₃ and EGF receptors into the newly formed focal contacts (Jones, et al., 1997b). Miyamoto and colleagues (Miyamoto, et al., 1996) have shown in fibroblasts that integrins associate with a wide variety of growth factor receptors including EGF, basic
fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) receptors. Moreover, the cytoplasmic tail of the EGF receptor binds directly to actin filaments and depolymerization of actin, with cytochalasin D, attenuates signaling through EGF and PDGF receptors (den Hartigh, et al., 1992; Diakonova, et al., 1995; Gronowski and Bertics, 1995). Indeed, in the absence of integrin clustering, ineffectual signaling through growth factor receptors bound with ligand is observed, whereas, complete growth factor signaling, involving the activation of mitogen-activated protein kinases (MAPK) including the extracellular signal-related kinase (ERK), requires focal adhesion contact formation (Renshaw, et al., 1996).

In addition, as observed in mammary epithelial cells, gene transcription is dependent upon the state of chromatin organization (Myers, et al., 1998; Bissell, et al., 1999), which is directly influenced by the rearrangement of the nuclear matrix by the contiguous actin cytoskeleton (Boudreau and Jones, 1999). Thus, focal adhesion contact formation may regulate gene transcription by altering the accessibility of transcription factors and other signaling factors, like ERK, to their target genes (Boudreau and Jones, 1999). For example, the loss of transcription factor access to anti-apoptotic protein encoding genes, as a result of focal contact disassembly, may initiate the apoptotic cascade.

An additional possibility is that disassociation of the β3 integrin subunits induces a pro-apoptotic signal, such as the activation of a caspase family member, however, evidence for such a signal has not been forthcoming and surprisingly little is known about the existence of signals derived from unligated integrin receptors.

Taken together, these studies suggest that the MMP-induced expression of TN mediates SMC survival by downstream intracellular signaling events initiated through the clustering of β3 integrins following ligation to TN. Based on the present studies, and our work in
organ culture using αvβ3 integrin blockade to induce a reduction in vessel wall thickness (Cowan, et al., 2000), the targeting of ECM survival ligands (Cowan, et al., 1999; Cowan, et al., 2000) or, once elucidated, the downstream pathways leading to smooth muscle cell apoptosis that are initiated by αvβ3 integrin unclustering, may contribute to a therapeutic arsenal for both pulmonary and systemic vascular disease.
CHAPTER FIVE

Complete Reversal of Fatal Pulmonary Hypertension in Rats by a Serine Elastase Inhibitor
ACKNOWLEDGEMENTS

Having orchestrated the study described in this chapter, I would like to note the contributions of summer students. Adrian Heilbut (summer '98) performed both the early trials as well as the one week timecourse study with elastase inhibitors including gavaging animals, measuring pulmonary artery pressures, making right ventricular hypertrophy measurements and doing barium-gelatin perfusions. Catherine Lam (summer '99) was responsible for morphometrically grading sections and performing additional measurements of right ventricular hypertrophy. Dr. Tilman Humpl (fall to summer '98-'99) carried out the extended two week timecourse of treatments and pressure measurements as well as grading sections and performing immunohistochemistry and tissue elastase assays. In addition, I would like to acknowledge contributions by other laboratories including Dr. Shinya Ito’s laboratory, which was responsible for measuring circulating elastase inhibitory activity, and Dr. Daniel Osmond’s laboratory, which performed all systemic pressure measurements.

INTRODUCTION

Progression of pulmonary hypertension is associated with elevated serine elastase activity and the proteinase-dependent deposition of the extracellular matrix smooth muscle cell (SMC) survival factor tenascin-C (Jones, et al., 1997a; Rabinovitch, 1998). Tenascin-C amplifies the response of SMCs to growth factors (Jones, et al., 1997b) which are also liberated through extracellular matrix proteolysis (Thompson and Rabinovitch, 1996). Recent organ culture studies, using hypertrophied rat pulmonary arteries, have shown that elastase inhibitors suppress tenascin-C, prevent proliferation and induce SMC apoptosis (Cowan, et al., 1999; Cowan, et al., 2000). This initiates complete regression of the hypertrophied vessel wall by a coordinated loss of cellularity and extracellular matrix.

We now show that when pulmonary hypertension, induced in rats by the toxin monocrotaline, is at an advanced stage, the progressively fatal disease process can be
reversed by oral administration of a peptidyl trifluoromethylketone which inhibits serine elastase activity. A two week treatment is sufficient to result in normalization of pulmonary artery pressure and regression of severe structural lesions in peripheral pulmonary vessels through coordinated SMC apoptosis and loss of extracellular matrix. While previous reports have shown that pulmonary hypertension can be reduced by concomitant treatment with agents such as L-arginine (Mitani, et al., 1997), this is the first study to document complete regression once malignant disease has become established.
MATERIALS AND METHODS

Experimental Design

Adult male Sprague-Dawley rats (250-300g) (Charles River Laboratories, St. Laurent, CN) were allocated to different experimental groups 21 days following a subcutaneous injection of saline or the alkaloid toxin monocrotaline (60mg/kg) (Sigma Chemical Co., St. Louis, MO) to induce pulmonary hypertension. The experimental groups included untreated animals, or those that received twice daily gavage tube feedings of one of two selective serine elastase inhibitors, ZD0892 or M249314 (240mg/kg/day) (kind gifts from Dr. C. Veale, Zeneca Pharmaceuticals, Wilmington, DW), or their administration vehicle 5% polyethylene glycol (PEG) or dimethylsulphoxide (DMSO) (Sigma). Animals were examined after 7 days of treatment (day 28). Based on initial findings, an additional treatment group was administered M249314 for 14 days (day 35). ZD0892 and M249314 are peptidly trifluoromethylketones with an N-terminal 4-(CH3O)C6H4CO group making them orally bioavailable serine elastase inhibitors (Edwards, et al., 1997). They are highly selective for neutrophil elastase (ki=-6.7nM) but, also, inhibit pancreatic elastase (ki=-200nM) and endogenous vascular elastase (Cowan, et al., 2000) but, not metallo, cysteine or other serine proteinases. Inhibitors were chosen to target the heightened elastase activity demonstrated in monocrotaline-induced vascular disease (Cowan, et al., 1999) and doses were chosen based upon our previous studies (Lee, et al., 1998). All protocols were approved by the Animal Care Committee of the Hospital for Sick Children.

Elastase Assays

Elastases were extracted from lung tissue, as previously described, (Lee, et al., 1998) and 10µg protein samples were analyzed for their ability to degrade BIODIPY fluorescein-conjugated bovine neck ligament elastin (EnzChek Elastase Assay Kit, Molecular Probes, Eugene, OR). Assays were performed using the assay kit protocol with extraction buffer as a negative control and porcine pancreatic elastase as a positive control. Inhibitory assays
were performed by challenging 3μl of rat serum, from an intracardiac bleed, with 1 unit of porcine pancreatic elastase.

**Survival Endpoints**

Criteria determined and set forth by the Animal Care Committee at the Hospital for Sick Children as 'humane endpoints' were monitored and rats meeting these criteria were required to be sacrificed, and counted as a loss in survival. These endpoints included a sustained bradycardia <100 beats/min, arterial oxygen tension <80%, and with abrupt weight loss >10% reduction in body weight/day for two days. Animals were evaluated for these criteria by the veterinary staff of our Animal Facility who recommended sacrifice without knowledge of the treatment group.

**Hemodynamic Measurements**

Direct pulmonary artery catheterization was performed and pressures measured (ES 1000 electrostatic recorder, Gould, Cleveland, OH) under isofluorane (Zeneca, Mississauga, ON) general anesthesia by a closed-chest technique described previously in detail (Rosenberg and Rabinovitch, 1988). Systemic blood pressure was determined by direct left carotid catheterization and measured by blood pressure analyzer (Statham DC pressure transducer, Chato Rey, PR; MacLab/8 data acquisition system, Lab Instruments, Castle Hill, Australia).

**Right Ventricular Hypertrophy**

The right ventricle (RV) was dissected from the left ventricle plus septum (LV+S) and weighed separately. The weight ratio of RV/(LV+S) was then calculated.

**Morphometric Assessments**

After the hemodynamic measurements were completed, lung tissue was prepared for morphometric analysis of the vasculature, using the barium-injection method previously
reported in detail (Rosenberg and Rabinovitch, 1988). Light-microscopic slides were analyzed by blinded observers as described (Rosenberg and Rabinovitch, 1988). Briefly, all barium-filled arteries >15 μm external diameter were assessed for the presence of muscularity and wall thickness of normally muscular arteries with external diameters in the ranges of 50-100μm and 101-200μm were measured as a percent of the lesser curvature (400x magnification).

**Immunohistochemistry and Detection of Apoptosis**

Immunohistochemistry, using techniques previously described (Cowan, et al., 1999), was performed on lung sections perfused with saline and fixed in 4% paraformaldehyde. For detection of PCNA, an anti-PCNA monoclonal antibody (1:100) (Dako) was used. To quantify apoptosis, TUNEL assays were performed with the Apoptag in situ detection system (Oncor Inc., Gaithersberg, MD). Nuclear morphology was examined by labeling with propidium iodide (2μg/ml). The relative number of proliferating and apoptotic cells was quantitatively assessed as a percent of total propidium iodide stained cells. Tenascin-C was identified with an anti-rat formaldehyde-fixed TN polyclonal antibody (1:100) (a generous gift from Dr. H. Erickson, Duke University Medical Center, Durham, NC). Estimates of total elastin were made using the Movat pentachrome stain in which elastin is identified with iron haematoxylin. The relative abundance of the extracellular matrix components examined were graded quantitatively using the Image-Pro Plus program for Macintosh (Media Cybernetics, Silver Spring, MD) as previously described (Cowan, et al., 1999). Briefly, the program performs planimetry and densitometry on positive staining above a uniform, 'background' cut-off. Subsequent multiplication of both the total area positively stained and the average density of staining provides a relative densitometric unit of the extracellular matrix protein being examined.
**Statistical Analysis**

Values from multiple experiments are expressed as mean±standard error and statistical significance was determined using one-way analysis of variance followed by Fisher's least significant difference test of multiple comparisons to establish individual group differences.
RESULTS AND DISCUSSION

Rats were injected subcutaneously with the toxin monocrotaline to induce an endothelial injury leading to severe pulmonary hypertension 21 days later. Then the rats were fed, by gavage, one of two peptidyl trifluoromethylketone serine elastase inhibitors, ZD0892 or M249314 or their respective administration vehicles. Circulating elastase inhibitory activity was assessed in plasma at various timepoints following gavage tube feeding of the inhibitor M249314 (Fig. 5.1A). The serum inhibitory activity increased to a level capable of inhibiting 2.5 units (per ml of serum) of purified porcine pancreatic elastase by 2 hours, 3.5 inhibitory units/ml by 6 hours, but began to decline at some later timepoint, reaching 1.2 units/ml by 12 hours when the rats were given the next dose. This sustained serine elastase inhibitory activity resulted in complete suppression of the monocrotaline-induced elastolytic activity observed in the lungs of vehicle or untreated rats (Fig. 5.1B). The latter was elevated 9 fold over that detected in normal lung tissue, at 21 days, and a further 8 fold at 28 days. Administration of either inhibitor, ZD0892 or M249314, reduced this activity to normal saline control levels, as assessed at 28 days as well as at 35 days.

Inhibition of serine elastase activity reversed the progressive mortality of untreated or vehicle treated rats, which started at 23 days following injection (Fig. 5.1C). Only 39% of untreated or vehicle treated rats survived to 28 days after monocrotaline, whereas 92% of rats treated for one week with either elastase inhibitor were alive. When treated with the elastase inhibitor M249314 for 2 weeks (day 35), the survival was still 86%, but by 30 days all the untreated rats had died.

That the cause of death in the vehicle treated or untreated group was related to progressive pulmonary hypertension, and that survival represented reversal of the disease, was reflected in hemodynamic assessments. By day 21 after monocrotaline injection, a two fold increase in mean pulmonary artery pressure from ~19mmHg to ~38mmHg was observed by direct
catheter study (Fig. 5.1D). There was a further elevation in mean pulmonary artery pressure to \( \sim 44 \text{mmHg} \) over the next week in untreated or vehicle treated rats. In contrast, a progressive reduction in pulmonary artery pressure was documented in conjunction with elastase inhibitor treatment such that by day 35, normal levels were observed (\( \sim 22 \text{mmHg} \)). This was a selective reduction in pulmonary artery pressure, as systemic arterial pressures were unchanged (data not shown). Reduced pulmonary artery pressure was associated with an arrest in the progression of right ventricular hypertrophy (Fig. 5.1E). This was not unexpected, since, in patients with pulmonary hypertension secondary to heart or lung disease, reversal of right ventricular hypertrophy lags behind a reduction in pulmonary artery pressure.

We next confirmed that the mechanism causing reversal of progressive pulmonary hypertension and arrest of right ventricular hypertrophy was related to the regression of severe structural changes in the peripheral pulmonary arteries. We quantified muscularization of distal pulmonary arteries by barium-gelatin perfusion, a technique which we have shown distends vessels evenly and results in reproducible assessments. The majority of pulmonary arteries between 15-50 \( \mu \text{m} \) in diameter are normally non-muscular arteries, as observed in saline-injected rats, with only a small proportion being muscularized, \( \sim 17\% \) (Fig. 5.2A). This muscularized subpopulation is expanded to \( \sim 65\% \) by 21 days and \( \sim 84\% \) by day 28 in monocrotaline-treated rats. Elastase inhibitor treatment induced progressive regression of this abnormal peripheral vascular muscularization, recovering the non-muscular artery pool and reducing the muscularized population to within the normal range (\( \sim 21\% \)) by day 35. In addition, normally muscularized pulmonary arteries showed regression of medial hypertrophy, as illustrated in Fig. 5.2B and 5.2C. Quantification of these changes in wall thickness were assessed for arteries in the ranges of 50-100 \( \mu \text{m} \) and 101-200 \( \mu \text{m} \) (Fig. 5.2D & E, respectively). Both populations of muscular pulmonary arteries exhibited an \( \sim 8 \) fold increase in thickness following monocrotaline-injection, which
was progressively reversed by the elastase inhibitors, reaching normal levels by day 35.
During progressive monocrotaline-induced pulmonary hypertension, there is a continuous reduction in the number of peripheral pulmonary arteries assessed as an increasing ratio of alveoli to arteries (Rabinovitch, 1998) (Fig. 5.2F). This has been attributed to closing off and resorption of peripheral vessels which could result from monocrotaline-induced endothelial injury and proteolytic degradation of basement membranes. The greater than two fold elevation in the ratio of alveoli to arteries (reduction in vessels), observed following monocrotaline, is arrested with one week of elastase inhibitor treatment (day 28) and a trend toward reversal is observed after 2 weeks (day 35).

The cellular mechanism accounting for the reversal of the monocrotaline-induced peripheral arterial muscularity with elastase inhibitors was investigated. Consistent with previous reports (Cowan, et al., 1999; Cowan, et al., 2000), deposition of the proproliferative matrix component tenasin-C, associated with medial hypertrophy at 21 days following monocrotaline-injection, correlated directly with marked vascular cell proliferation and inversely with apoptosis (remaining relatively absent) (Fig. 5.3A, E, I, M, Q & R). Together with tenasin-C, the presence of proliferation, reflected in cells positive for the proliferating cell nuclear antigen, was enhanced in both untreated and vehicle treated rats at 28 days (~1.8 fold increase relative to day 21), while apoptosis was absent or minimal (Fig. 5.3B-C, F-G, J-K, N-O, Q & R). Elastase inhibitor-induced regression of medial hypertrophy after one week, at the 28 day timepoint, was attributed to suppression of tenasin-C, and concomitant ~10 fold induction of apoptosis and ~5 fold (relative to day 21) suppression of proliferation (Fig. 5.3D, H, L, P, Q & R). In addition to loss of cellularity by apoptosis and despite elastase inhibition, reversal of hypertrophy of the vessel wall was associated with resorption of the pathogenic increase in extracellular matrix components, as reflected by measurements of elastin content (Fig. 5.3S).
The mechanism responsible for inducing elastase activity during the initiation and progression of pulmonary hypertension (Todorovich-Hunter, et al., 1992) may be related to the endothelial injury (Rosenberg and Rabinovitch, 1988) and loss of barrier function. We have shown in cell culture that serum factors can induce activity of an endogenous vascular elastase in SMCs by a mitogen activate protein kinase signaling mechanism (Thompson, et al., 1998) involving increased nuclear expression of the transcription factor AML-1 (Wigle, et al., 1998). In cultured pulmonary artery SMCs, heightened elastase activity can release extracellular matrix-stored growth factors, such as FGF-2 (Thompson and Rabinovitch, 1996). The SMC proliferative response to growth factors can be amplified by elastase-mediated induction of the glycoprotein tenascin-C. We have shown that proteolysis of collagen, and exposure of RGD binding sites (Kafienan, et al., 1998), will induce β3 integrin-dependent transcription of tenascin-C (Jones, et al., 1999). Tenascin-C, subsequently, clusters β3 integrins as well as growth factors and facilitates their phosphorylation upon ligation (Jones, et al., 1997b).

Conversely, apoptosis of SMCs is likely induced through suppression of tenascin-C expression resulting from inhibition of elastase activity. Inhibition of elastase may, by arresting ongoing collagen degradation, prevent the β3 integrin-dependent signal which induces tenascin-C, and loss of SMC interaction with tenascin-C may initiate the apoptotic cascade. Indeed, apoptotic SMCs in culture are rescued by addition of exogenous tenascin-C and subsequent β3 integrin ligation (Jones, et al., 1997b). An αvβ3 integrin survival signal is further supported by studies in which blocking endothelial cell αvβ3 integrin interactions, with either cyclic peptides or functional blocking antibodies, induces apoptosis and vessel loss (Brooks, et al., 1994b; Choi, et al., 1994). The αvβ3 integrin pathway in angiogenic endothelial cells appears to suppress p53 and the cyclin-dependent kinase inhibitor p21WAF1/CIP1 (Stromblad, et al., 1996), and sustain ERK activity (Eliceiri, et al., 1998). Thus, αv knockout mice exhibit an embryonic lethal phenotype (Bader, et al., 1998).
The importance of this pathway in remodeling vessels (Brooks, et al., 1994b) is reflected in our studies in organ culture since SMC apoptosis only occurred in hypertensive actively remodeling and not in normal vessels (Cowan, et al., 1999). That apoptosis was accompanied by loss of the excess extracellular matrix synthesized and deposited during the remodeling process seemed unexpected in the presence of proteinase inhibitors. It is possible that there is ongoing phagocytosis and intracellular degradation of the matrix by enzymes, produced by apoptotic or viable SMCs, that are unaffected by the elastase inhibitors used (Cowan, et al., 2000).

Taken together, these findings indicate that heightened serine elastase activity is both pivotal to the progressive proliferative response resulting in excessive muscularity of distal vessels and is also necessary for survival of these SMCs. Selective elastase inhibitors can, therefore, induce apoptosis which, in association with resorption of the extracellular matrix, leads to regression of the hypertrophied vessel wall. This is directly related to normalization of pulmonary artery pressure, arrested right ventricular hypertrophy, and improved survival, thus offering a novel and potentially clinically applicable therapeutic approach to treating severe pulmonary hypertension.
Figure 5.1

Elastase inhibitory activity assays assess circulating plasma inhibitory activity in A, while changes in elastolytic activity in rat lung homogenates are measured with fluorogenic elastin in B. Changes in survival of monocrotaline-injected, treated and untreated rats is plotted in C. Animals either died or were sacrificed in accordance with humane endpoints set forth by the Animal Care Committee. Results of direct pulmonary artery catheterization are expressed as mean pulmonary artery pressure in D, and the ratios of the right ventricle (RV) over the left ventricle plus septum (LV+S), as an index of right ventricular hypertrophy, are displayed in E. These graphs indicate that inhibition of monocrotaline-induced elastolytic activity results in survival, and is related to a reversal of pulmonary hypertension and arrest in progression of right ventricular hypertrophy. Letter designations indicate Untreated, Vehicle treated, and Inhibitor treated rats with either ZD0892 or M249314; data points and bars = mean + SD of n = 3 in A and B, mean + SEM of n = 6 for D and E, and in C n = 11 for I², 16 for I¹, 34 for V, and 27 for U; * = p<0.05 compared to saline-injected controls and † = p<0.05 compared to day 21 monocrotaline-treated rats.
Figure 5.2

Morphological assessments were performed on barium-perfused arteries. The presence and degree of muscularization in normally non-muscular pulmonary arteries (15-50μm) are displayed as a percent of total pulmonary arteries in A (muscularized arteries are compared by statistical analysis). Changes in medial thickness as a percent of the total diameter in pulmonary arteries which are normally muscular, between 50-100μm and 101-200μm, are illustrated in B (vehicle) and C (inhibitor) and quantified in D and E. The ratio of pulmonary arteries to alveoli is graphically displayed in F. Thus, elastase inhibition reverses pulmonary artery muscular changes and prevents further loss of small vessels. Letter designations indicate Untreated, Vehicle treated, and Inhibitor treated rats with either ZD0892 or M249314; bars = mean + SEM of n = 4-7; scale bar = 10μm; * = p<0.05 compared to saline-injected controls and † = p<0.05 compared to day 21 monocrotaline-treated rats (in F compared to monocrotaline day 28).
Figure 5.3

Representative photomicrographs of saline perfused, Movat pentachrome stained pulmonary arteries are shown in A, 21 day monocrotaline, B, 28 day monocrotaline untreated, C, 28 day monocrotaline vehicle treated, and D, 28 monocrotaline inhibitor treated. Directly below (E-H) are corresponding representative pulmonary arteries following tenascin-C immunohistochemistry (positive brown peroxidase staining is indicated with arrows), in situ TUNEL assays identifying apoptosis are further below (I-L) (examples of TUNEL positive vascular cells are indicated with white arrows) and proliferating vascular cells are shown in the lowest row of photomicrographs (M-P), by immunohistochemistry for the proliferating cell nuclear antigen (PCNA) (dark nuclei are PCNA positive cells). Percent cell counts of TUNEL and PCNA positive cells are displayed graphically in Q and R, respectively, and densitometric quantification of elastin is provided in S. Thus, elastase inhibition arrests tenascin-C accumulation and proliferation, and induces apoptosis and loss of extracellular matrix, e.g. elastin. Letter designations indicate Untreated, Vehicle treated, and Inhibitor treated rats with either ZD0892 or M249314; bars = mean + SEM of n = 4; scale bar = 5μm; * = p<0.05 compared to saline-injected controls and † = p<0.05 compared to day 21 monocrotaline-treated controls.
SUMMARY AND FUTURE DIRECTIONS

In this thesis, we established the pathophysiologic significance of heightened serine elastase and MMP activity as well as increased αvβ3 integrin survival ligands, like tenascin-C, in the progression of PVD. In so doing, we observed that MMPs and serine elastases function in a co-dependent manner and that vascular SMCs exhibit pluripotentiality with respect to ECM survival ligand usage. Moreover, disruption of a pathway which depended on increased proteolysis and ligation and clustering of αvβ3 integrins, compromised SMC survival, and this resulted in disease reversal by a coordinated loss of cellularity and extracellular matrix.

Based on evidence that tenascin-C was progressively increased with the development of monocrotaline-induced PVD and that, in cell culture, tenascin-C amplified the proliferative response of SMCs to growth factors (Jones and Rabinovitch, 1996), we sought to establish whether upregulation of tenascin-C occurred in clinical PVD, thereby making it a potential therapeutic target. By characterizing the expression of tenascin-C in pulmonary arteries on lung biopsy specimens from children with pulmonary hypertension as a result of uncorrected congenital heart defects, we documented that tenascin-C deposition correlated with increasing grade of lesion and co-distributed with growth factors and proliferating SMCs. Additional observations indicated that the intimal lesions were also associated with heightened expression of the pro-migratory glycoprotein fibronectin in a distribution gradient most intense in the subendothelium. This feature has also been reported in the intimal cushions of the ductus arteriosus (Boudreau and Rabinovitch, 1991; Boudreau, et al., 1991; Boudreau, et al., 1992) and in neointimal formation in coronary arteries following cardiac transplantation (Clausell, et al., 1993b; Clausell, et al., 1993a; Molossi, et al., 1995a; Molossi, et al., 1995b; Molossi, et al., 1995c).
Taken together, these studies suggested that targeting tenascin-C might halt SMC proliferation and disease progression associated with subsequent fibronectin-dependent migration. In addition, as tenascin-C was required for survival of SMCs in culture (Jones, et al., 1997b), the possibility existed that a reduction in tenascin-C might cause cell loss and reverse vessel remodeling towards normal.

In previous cell culture studies with PA SMCs, tenascin-C was regulated by MMPs and mechanical stress (Jones, et al., 1997b; Jones, et al., 1999). Specifically, on attached collagen gels, MMPs upregulate tenascin-C leading to SMC proliferation, whereas on floating collagen, reduced MMPs suppress tenascin-C and induce SMC apoptosis. To address the possibility of vascular regression by tenascin-C suppression, we investigated the response of SMCs in the whole vessel by comparing normal main PAs derived from juvenile pigs embedded in attached or floating collagen gels for 8 days. Normal porcine PAs in attached collagen gels were characterized by increasing activity of MMPs-2 and -9 and tenascin-C deposition, whereas a reduction in these features was observed in PAs on floating collagen. The deposition of tenascin-C within the vessel wall formed a patchy pattern in which tenascin-rich foci were associated with proliferation and tenascin-poor areas with apoptosis. However, no significant difference in wall thickness was observed when comparing attached and floating conditions.

We then speculated that, as normal vessels are relatively resistant to changes in tenascin-C, proliferation and apoptosis leading to morphological differences, hypertensive vessels, already in a state of active remodeling, may be more responsive to manipulation of tenascin-C by attached versus floating conditions. Furthermore, since serine elastases are increased in hypertensive PAs (Todorovich-Hunter, et al., 1992), they may promote a heightened activation of MMPs (Okada, et al., 1988; Nagase, et al., 1990; Matsumoto, et al., 1992), or might directly enhance the upregulation of tenascin-C by growth factor release (Rettig, et al.,
1994) or ECM proteolysis (Kafitenan, et al., 1998), as shown for MMPs (Jones, et al., 1997b). Pulmonary arteries from both normal and hypertensive rats, at 21 days following monocrotaline injection, were then similarly cultured while embedded in attached and floating gels. While normal rat PAs were similar to piglet vessels, hypertrophied rat PAs showed an amplified response. Increased elastase, MMP-2, tenasin-C and elastin deposition, and SMC PCNA positivity correlated with progressive medial thickening on attached collagen, whereas reduced MMP-2, elastase, tenasin-C, and induction of SMC apoptosis accompanied regression of the thickened media on floating collagen.

Since regression was associated with reduced proteinase activity, we investigated whether direct elastase or MMP inhibition would recapitulate this response. Hypertrophied rat PAs in organ culture, which progressively thicken in association with cell proliferation and matrix accumulation, were induced to regress by inhibiting either serine elastases or MMPs. This regression was associated with reduced tenasin-C, suppression of SMC proliferation, and induction of apoptosis.

Interestingly, inhibition of serine elastases also suppressed MMP activity, attributable to MMP-2, and MMP inhibitors reduced elastase activity. These results suggest that the two classes of enzymes may be coordinately regulated in our culture system and potentially in the intact vessel wall. While we have speculated that this regulation may be through the proteolytic activation of MMPs by serine elastases (Okada, et al., 1988; Matsumoto, et al., 1992) as well as elastase-mediated degradation of native MMP inhibitors (Okada and Nakanishi, 1989; Itoh and Nagase, 1995) and, conversely, the inactivation of native serine elastase inhibitors by MMP proteolysis (Chin, et al., 1985; Kataoka, et al., 1999), preliminary studies have suggested that another form of regulation may also be involved. We have shown, preliminarily, by western immunoblot, that inhibition of elastase activity leads to a decrease in the amount of both latent and active MMP-2. Thus, serine elastases
may regulate MMP-2 at a transcriptional or post-transcriptional level. Transcriptional regulation may be through elastase liberation of growth factors (Thompson and Rabinovitch, 1996; Aho, et al., 1997; Miyake, et al., 1997) and cytokines (Luca, et al., 1997; Wu, et al., 1997) or through the production of ECM peptide fragments (Landeau, et al., 1994; Tyagi, et al., 1996) which stimulate MMP expression. In rabbit synovial fibroblasts, collagenase and stromelysin gene expression is induced when fibronectin fragments bind the integrin receptors α5β1 and α4β1 (Werb, et al., 1989; Huhtala, et al., 1995).

To further address whether MMP expression is induced by elastase activity and the mechanism involved, SMCs in culture could be spiked with serine elastases like human leukocyte elastase, or stimulated to produce the endogenous vascular elastase with serum-treated elastin, and metabolic labeling could be used to examine changes in newly synthesized MMP-2 protein. The level of regulation could also be examined by northern blotting with nuclear run-off assays to assess if increased transcription is involved. If so, characterization of the MMP-2 promoter could be done with promoter-reporter (CAT) constructs and nested deletions to identify minimal responsive sequences. To determine whether MMP-derived factors stimulate serine elastase activity, further characterization of the endogenous vascular elastase is required, that is, it will be necessary to clone the gene.

While we have focused on the involvement of serine elastases and MMPs in the development and progression of vascular thickening in our model, and have shown that inhibition of either MMPs or elastases is sufficient to induce reversal of thickening, recent studies have suggested that other enzymes may play pivotal roles in vascular pathobiology. For example, Sukhova et al (Sukhova, et al., 1998) have reported that the cysteine proteinases cathepsin K and S are markedly upregulated by SMCs and macrophages at sites of atherosclerotic intimal formation and account for much of the elastin degradation observed in atheromas. Thus, despite a lack of macrophages, we cannot, at present, exclude
the possibility that SMC-produced cathepsins are part of the proteolytic response in the hypertrophied pulmonary arteries in organ culture. This would, however, appear unlikely since elastase activity extracted from freshly isolated hypertrophied pulmonary arteries after monocrotaline was not reduced by cysteine proteinase inhibitors (Todorovich-Hunter, et al., 1992).

In studies with MMP and elastase inhibitors, as with the attached and floating collagen gel model, hypertensive arteries responded to treatment while normal arteries were relatively refractory, at least in terms of changes in wall thickness. Selective responsivity has also been reported in studies inducing regression of angiogenic but not normal vessels (Brooks, et al., 1994a; Brooks, et al., 1994b; Brooks, et al., 1995; Drake, et al., 1995), and may be related to the upregulation of integrin receptors, like the αvβ3 (Stromblad, et al., 1996; Boudreau, et al., 1997; Eliceiri, et al., 1998). It would be important to further characterize the phenotype of SMCs in PVD to determine the features that specifically contribute to increased susceptibility to apoptosis in response to proteinase inhibition and loss of survival ligands. Differences in integrin activation, localization, and/or expression in response to growth factors (Boudreau, et al., 1997) may be important. By culturing SMCs from normal and hypertensive rats with proteinase inhibitors in the presence and absence of EGF, we would better identify how changes in growth factor receptor signaling collaborates with altered integrin signaling in inducing apoptosis. Moreover, dominant negative integrins and growth factor receptors could then be used to further tease out the relative contributions of each pathway.

As tenascin-C suppression appeared critical to apoptosis (Jones, et al., 1997b) and regression of medial hypertrophy associated with MMP and serine elastase inhibition, we selectively targeted tenascin-C by transfecting PAs with antisense/ribozyme constructs. This approach induced SMC apoptosis and arrested progressive vascular thickening, but
failed to induce regression. We then related this finding to a concomitant expansion of a SMC population which produced an alternative cell survival αvβ3 ligand, osteopontin (Liaw, et al., 1995; Scatena, et al., 1998), in response to pro-proliferative cues provided by a proteolytic environment. Indeed, exogenous osteopontin could rescue SMCs from MMP inhibitor-induced apoptosis in cell culture studies. Consistent with this, immunohistochemical staining for osteopontin in proteinase inhibitor-treated PAs revealed that, like tenascin-C, osteopontin was similarly suppressed.

During this study, it was very apparent that vascular SMCs had considerable heterogeneity, as previously reported by Stenmark and coworkers (Frid, et al., 1994; Wohrley, et al., 1995; Frid, et al., 1997; Dempsey, et al., 1998), and exhibited pluripotentiality with regards to usage of ECM survival ligands. While tenascin-C and osteopontin were examined, changes in other ECM cell survival factors and αvβ3 integrin ligands, like thrombospondin, vitronectin, laminin or degraded collagen were not analyzed (Cheresh, 1991). Furthermore, other isoforms of tenascin, like tenascin-R, -X or -Y, might have been upregulated as this was suggested to account for the lack of a phenotype in tenascin-C knockout mice (Mackie, 1997). Therefore, these factors, which might also contribute to SMC survival during the progression of PVD, could be characterized in rat hypertensive arteries, in the presence and absence of tenascin-C antisense treatment. The characterization of SMC subpopulations on the basis of survival ligand usage would be a particularly interesting research focus that could use antisense constructs, like the one we produced for tenascin-C, to selectively remove specific subpopulations while amplifying others. Any identified SMC subpopulations could then be correlated with those previously described, that is, on the basis of morphological characteristics, growth capacity and/or meta-vinculin expression (Frid, et al., 1997).
The mechanism accounting for the upregulation of osteopontin by tenascin-C antisense is also of interest. Immunofluorescence double-labeling for tenascin-C and osteopontin suggested that while some cells produce either tenascin-C or osteopontin, another population may produce both and switch, following loss of tenascin-C by antisense, to the sole usage of osteopontin. Uncovering the mechanism by which a cell recognizes that it must upregulate an alternative survival ligand, as it is losing another, is a challenging project. One possibility is that there is feedback through the products of ongoing proteolysis. For example, both tenascin-C and osteopontin are upregulated by growth factors (Pearson, et al., 1988; Rodan, et al., 1989; Rettig, et al., 1994; Wang, et al., 1996; Yamamoto, et al., 1997) which are liberated when matrix is degraded (Thompson and Rabinovitch, 1996), as well as integrin-mediated signals which use common signaling pathways. However, as downregulation of tenascin-C occurs by antisense-mediated degradation of the tenascin-C mRNA, the reciprocal upregulation of osteopontin may be related to common post-transcriptional mechanisms, like the increased availability of factors that bind to common mRNA stability elements or elements involved in translation efficiency, as has been shown for collagen (Diaz and Jimenez, 1997), fibronectin (Zhou, et al., 1997; Zhou and Rabinovitch, 1998) and elastin (Johnson, et al., 1995; Hew, et al., 1999). It will be necessary, first, to document the level at which upregulation of osteopontin occurs in response to tenascin-C antisense. If it appears to be post-transcriptional, identifying whether increased osteopontin mRNA stability or translational efficiency is involved would follow, and then an examination of the untranslated region of the mRNA in both tenascin-C and osteopontin for common cis elements and potential trans-acting factors would be appropriate.

As tenascin-C and osteopontin ligate SMCs via αvβ3 integrins and are both suppressed by proteinase inhibition, we documented that αvβ3 integrin receptor blockade would also induce apoptosis and regression in hypertrophied arteries. We further delineated in cell
culture that ligation to these integrins was 'integral' to SMC survival. We showed, in a rat aortic SMC line, that in the absence of MMP inhibition, SMCs can be induced to apoptose with antibodies specific to either the αvβ3 or β3 integrin. This was accompanied by the observation that while β3 integrins are present in focal adhesion-like formations in the non-apoptotic condition, induction of apoptosis, by MMP inhibition, was associated with their unclustering. This suggested that it may be the clustering of β3 integrins that supports survival. Indeed, SMCs can be rescued from MMP-inhibitor induced apoptosis through the artificial clustering of β3 integrins using β3-directed antibodies with cross-linking antibodies. However, the downstream intracellular signaling events that promote survival will require further elucidation.

In other cell culture systems, with fibroblasts and epithelial cells, integrin-mediated apoptosis involves FAK and downstream kinases whose targets are components of the system that controls apoptosis, like BAD (Datta, et al., 1997), caspase-9 (Cardone, et al., 1998), p53 (Illic, et al., 1998), bcl-2 (Zhang, et al., 1995) and others (possible effects of β3 integrin clustering versus unclustering are summarized in Figure 6.1). In future studies, it would be interesting to determine whether there are changes in the expression of the IAP family of caspase inhibitors (Deveraux, et al., 1997; Roy, et al., 1997), which are upregulated by NF-κB signaling from αvβ3 integrins (Erli, et al., 1999). Failure to send this anti-apoptotic signal may then initiate apoptosis. Alternatively, loss of integrin ligation might instead be associated with a pro-apoptotic signal, but little is known about the existence of such a signal. Since induction of apoptosis has been often related to a conflict in cell cycle signals (Boudreau, et al., 1996), it is also possible that apoptosis results from the inability to deliver an appropriate growth factor signal because co-clustering with
Figure 6.1

Schematic representations of the consequence of β3 integrin clustering versus unclustering to apoptosis. As described in the text, clustering of β3 integrins could suppress apoptosis through the activation of kinase cascades, involving focal adhesion kinase (FAK), phosphoinositide 3-OH kinase (PI3 kinase), and protein kinase B/Akt (PKB/Akt), by the accumulation of tyrosine phosphorylated (Tyr-P) proteins at focal adhesion contacts and which ultimately phosphorylate (P) and inactivate pro-apoptotic factors like BAD and caspase-9. Integrin-mediated intracellular survival signals have also been reported to include upregulation of anti-apoptotic factors, like inhibitor of apoptosis protein (IAP)-1 and Bcl-2, and these may require coordinate signaling through growth factor receptors, like epidermal growth factor receptor (EGF-R). Focal adhesion contact formation, as a result of integrin clustering, is hard-wired, through actin stress fibres, to the nuclear matrix. As changes in nuclear structure regulate gene transcription, integrin clustering may be permissive to transcription of anti-apoptotic genes via enabling access of transcriptional regulatory factors (TFs). Alternatively, unclustered β3 integrins may be associated with an as yet uncharacterized pro-apoptotic signal, prevent signaling required for production of anti-apoptotic factors, or fail to extend the nuclear matrix and thereby prevent access of transcriptional regulatory factors to genes encoding anti-apoptotic proteins. Moreover, as integrin and growth factor receptors are functionally related and co-distribute at focal adhesions, the inability to coordinate growth factor signals through focal contacts, which are dependent on integrin clustering, may result in a conflict in cell cycle cues leading to an apoptotic default pathway.
Clustered β3 integrins could:

1. Activate kinase cascades (FAK, PI3 Kinase, & PKB/Akt) that inactivate pro-apoptotic factors, ie. BAD, Caspase-9, etc.

2. Send signals that increase anti-apoptotic factors, ie. IAP-1, Bcl-2, etc.

3. Induce changes in the nuclear matrix that are permissive to transcription (TFs) of genes that may be anti-apoptotic.

Unclustered β3 integrins could:

1. Send an as yet uncharacterized pro-apoptotic signal or prevent inactivation of pro-apoptotic factors.

2. Prevent signals necessary for anti-apoptotic factors, e.g., IAP-1.

3. Alter nuclear matrix and prevent transcriptional access to anti-apoptotic genes.

4. Cause a conflict in cell cycle cues through an inability to coordinate growth factors signals.
integeins at focal adhesion contacts has been disrupted (Jones and Rabinovitch, 1996; Miyamoto, et al., 1996; Renshaw, et al., 1996; Jones, et al., 1997b).

Yet another possibility exists. As the actin cytoskeleton connects ECM ligation through focal contacts with the nuclear matrix, there may be a functional consequence to the nuclear architecture resulting from a change in ECM contact (Bissell, et al., 1999; Boudreau and Jones, 1999; Stein, et al., 1999). Indeed, the chromatin and histone structure, influenced by the nuclear matrix, regulates gene transcription (Myers, et al., 1998). Thus, future studies aimed at delineating the mechanism involved in β3 clustering-mediated control of SMC apoptosis might include examining the effects of integrin clustering and focal contact formation on apoptosis-related gene transcription. That is, a comparison of the changes in gene regulation, with particular interest in genes encoding apoptosis control factors, by microarray screening of SMCs under apoptotic conditions, in the presence and absence of integrin cross-linking, may be very informative.

A consistent and provocative observation is that regression of vascular hypertrophy is achieved through a coordinated loss of both the cellular and matrix components of the vessel wall. While we have attributed the mechanism of SMC apoptosis to loss of survival ligands, ECM resorption occurs despite suppression of classical matrix degrading enzymes, MMPs and serine elastases, and is, in actuality, promoted by direct inhibition of these proteinases. Extracellular matrix resorption, shown in our studies using elastin as a hallmark, may be through phagocytosis and intracellular degradation by SMCs, as described for gingival fibroblasts (Sawada and Inoue, 1997), or may involve the production, secretion or activation of proteinases within the microenvironment at the cell surface of normal SMCs, in response to stimuli provided by apoptotic cells. Alternatively, as apoptosis itself is associated with the activation of various intracellular enzymes (Cohen, 1997; Barinaga, 1998; Kidd, 1998), loss of plasma membrane integrity and release of
intracellular components (Ormerod, et al., 1993; Lang, et al., 1998), it is tempting to speculate that these enzymes may be externalized or released into the extracellular compartment and account for the observed proteolysis. While it has not been established whether proteinases, like the caspase family of enzymes activated during the course of apoptosis (Cohen, 1997), are released into the extracellular compartment, members of this family, like Mch2α (caspase-6), degrade the nuclear matrix (Takahashi, et al., 1996) and may, therefore, also be capable of ECM proteolysis. In addition, as the caspases are cysteine proteinases (Villa, et al., 1997), they would not be targeted by the inhibitors used in our studies. Indeed, in ongoing work not presented in this thesis, we have preliminarily shown that apoptotic populations of SMCs exhibit enhanced collagen degradation in culture and increased elastolytic activity, associated with their cell membrane fraction. Moreover, activity of caspases -2, -3, -4 and -6 have been monitored in this cell fraction and the elastolytic activity can be largely attenuated with Boc-D-Fmk, a general caspase inhibitor, but not with serine elastase or MMP inhibitors. While additional studies are required to immunolocalize potential ECM-degrading caspases to the outer leaflet of the plasma membrane on apoptotic SMCs and to directly implicate caspases in ECM degradation, such as the use of recombinant caspases in in vitro elastase or collagenase assays, this work suggests that the activation, release or externalization of specific caspases by apoptotic SMCs, may facilitate vascular lesion regression through the resorption of ECM.

As we have explored the mechanisms by which to induce SMC apoptosis and have shown, in organ culture, that this process can be harnessed to reverse vascular disease, we pursued this approach in the intact animal. Using orally bioavailable peptidyl trimethylfluoroketone serine elastase inhibitors, which induce regression in organ culture, we treated rats with advanced pulmonary vascular disease 21 days following injection of monocrotaline. A one week treatment resulted in 92% survival versus 39% in untreated rats. Pulmonary artery pressure and muscularization were reduced by myocyte apoptosis and loss of ECM,
specifically elastin and tenascin-C. After 2 weeks, PA pressure and structure normalized and survival was 86% versus none in controls.

As this study reproduces our organ culture findings with the serine elastase inhibitor, it is not surprising that other organ culture models can be extended to the intact animal. Indeed, based on our work showing similar regression of hypertrophy following alterations in mechanical stress in the attached and floating collagen gel system, further work in the laboratory has shown that stress unloading of PAs in a diseased lung from a monocrotaline-treated rat, following transplant of that lung into a normotensive rat, also induces complete regression of structural and functional abnormalities within two weeks (O'Blenes, et al 2000, manuscript in preparation). Thus, one might suggest that αvβ3 integrin blockade will similarly reverse monocrotaline-induced vascular disease. These studies are now ongoing in an attempt to establish an arsenal of therapeutic options to treat PVD. However, some issues remain to be addressed. For example, will PVD redevelop following cessation of treatment? Preliminary anecdotal results, specifically the continued survival (now over eight months) of two monocrotaline-treated rats receiving only one week of elastase inhibitor treatment, suggest that PVD will not reoccur and that, perhaps, as short a time as one week of therapy may initiate the reversal process. A complete study is, of course, necessary to substantiate these observations.

While pulmonary hypertension and arterial muscularization were reversed by elastase inhibitor treatment, progressive right ventricular hypertrophy and loss of peripheral arteries was only prevented. We have speculated that there may be a delayed response. This is based upon a slight but non-significant trend toward a reduction in right ventricular hypertrophy and, the observation that in patients following congenital heart defect repair, regression of right ventricular hypertrophy lags behind the reduction in pulmonary artery pressure. On the other hand, recovery of small pulmonary arteries likely involves an
angiogenic response that requires MMP and, potentially, elastase activity as well as ligation of αvβ3 integrins for the migration of endothelial cells (Haas and Madri, 1999). As suppression of these features was targeted by our approach, revascularization may not be possible during therapy. Thus, while normalization of pulmonary pressure is observed under resting conditions, a loss of vascular reserve may induce pulmonary hypertension with exercise. It is, however, conceivable that, following the cessation of treatment, initiation of angiogenesis may take place, and this will be addressed in future studies.

The potential to induce regression of human pulmonary vascular lesions relies on similarities between experimental models and clinical tissue. Indeed, at the outset, we have established that tenascin-C, associated with proliferating SMCs, is upregulated in human lesions similar to monocrotaline-induced disease. However, the targets of successful therapies in the monocrotaline model have not been addressed in the clinical arena, with the exception of a reduction in mechanical stress, by surgical correction of congenital heart defects, which does result in a reversal of structural pulmonary abnormalities (Rabinovitch, et al., 1984; Haneda, et al., 1994). Specifically, for serine elastase inhibitors to be used as adjunctive therapy for PH patients, the presence of an active serine elastase in these patients should be established. Recent work in our laboratory has preliminarily shown that in lung tissue homogenates from primary and secondary PH patients, compared to normal tissue, a greater proportion of the detected elastolytic activity is due to a serine elastase. Moreover, preliminary immunohistochemistry of lung biopsies from primary PH patients taken prior to transplantation, has indicated an upregulation of the putative transcription factor for the vascular serine elastase, AML-1.

We have begun to address the potential for regression of established human pulmonary vascular lesions by the collection and culture of diseased pulmonary arteries from patients with PH at the time of lung transplantation. These preliminary organ culture studies
suggest that proteinase inhibition may begin to reverse the chronically-developed intimal-based lesions observed in these patients and, thus, clearly warrant further investigation. With confirmation of these findings, the use of serine elastase inhibitors in human PH patients would be warranted and may impact on the progression of this chronic disease process. The design of highly selective agents, however, awaits the further characterization of the enzyme. Also, further characterization of human biopsy samples for the presence of MMPs (specifically MMP-2), whose activity has not yet been assessed in clinical tissue, as well as osteopontin and β3 integrins, would provide additional evidence that the pathway that we have elucidated in rats is relevant to the human condition despite significant differences in pathophysiology.

The severity, rate of progression and potential for reversibility of PH differs depending on the etiology. For example, the difference between the reversibility of hypoxia-induced PVD and irreversibility of monocrotaline-induced disease may be the absence versus presence of a sustained increase in serine elastase activity (Maruyama, et al., 1991; Todorovich-Hunter, et al., 1992). That is, while serine elastase activity remains elevated in monocrotaline-induced disease, only a transient increase in elastase activity is detected during hypoxia-induced PH in rats. While this may initially suggest that serine elastase inhibition may have only limited utility in reversing established hypoxia-induced PVD, independent of removing the hypoxic influence, clinical pathogenesis is believed to involve sequential insults to the vasculature which may be associated with repeated induction of elastase. In this case, elastase inhibition may prove effective prophylactically in arresting disease progression, as shown in hypoxic rats given elastase inhibitors at the onset of hypoxia (Maruyama, et al., 1991). Additional comparative experiments addressing other features associated with elastase activity in rats exposed to hypoxia, such as MMP activation, upregulation of SMC survival ligands and availability of SMC growth factors, may elucidate why these animals
exhibit a stabilization of PVD during chronic hypoxic exposure and, in normoxia, spontaneous disease regression.

While considerable studies remain to be performed, taken together, our data demonstrate a critical role for \( \beta 3 \) integrin ligating ECM survival factors, like tenascin-C, in the progression of PVD. Regulating the availability of such factors, through the control of proteinase activity or accessibility of their signaling receptors, may offer novel therapeutic options to treating and reversing this disease. Moreover, the parallels established between the behaviour of primary cultured PA smooth muscle cells and the aorta smooth muscle cell line suggest that these strategies may also be of value in systemic vascular disease.
REFERENCES


Chung CY, Erickson HP. Cell surface annexin II is a high affinity receptor for the alternatively spliced segment of tenasin-C. *J. Cell Bio.* 1994; 126:539-548.


Halpert I, Sires UI, Roby JD, Potter PS, Wight TN, Shapiro SD, Welgus HG, Wickline SA, Parks WC. Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. Proc Natl Acad Sci USA 1996; 93:9748-53.


Jones PL, Crack J, Rabinovitch M. Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the αvβ3 integrin to promote epidermal growth factor receptor phosphorylation and growth. *J. Cell Biol.* 1997b; 139:279-293.


