

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Smooth Muscle Cell Interaction with Fibrin-A Possible Mechanism
for Vessel Narrowing During Atherosclerosis

by

Karen O Yee


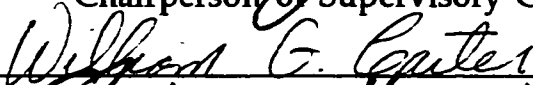
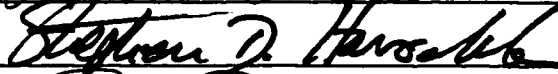
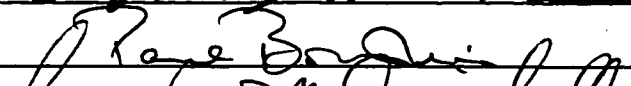
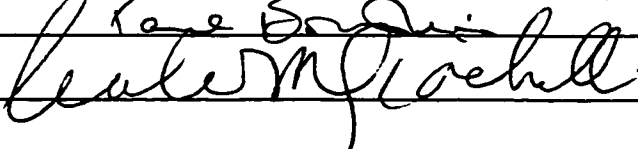
A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

University of Washington

1998

Approved by


Chairperson of Supervisory Committee





Program Authorized

to Offer Degree Molecular and Cellular Biology/FHCRC

Date November 18, 1998

UMI Number: 9916738

**UMI Microform 9916738
Copyright 1999, by UMI Company. All rights reserved.**

**This microform edition is protected against unauthorized
copying under Title 17, United States Code.**

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

In presenting this dissertation in partial fulfillment of the requirements for the Doctoral Degree at the University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying of this dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dissertation may be referred to University Microfilms, 1490 Eisenhower Place, P.O. Box 975, Ann Arbor, MI 48106, to whom the author has granted "the right to reproduce and sell (a) copies of the manuscript in microform and/or (b) printed copies of the manuscript made from microform."

Signature Karen O Yee

Date November 18, 1998

University of Washington

Abstract

Smooth Muscle Cell Interaction with Fibrin-A Possible Mechanism
for Vessel Narrowing During Atherosclerosis

by Karen O Yee

Chairperson of the Supervisory Committee
Professor Stephen M. Schwartz
Department of Pathology

Atherosclerosis is a disease that causes lumen narrowing in the vasculature. Recent studies suggest that integrin antagonists may inhibit narrowing by preventing cell interaction with the extracellular matrix in the plaque. A number of *in vitro* assays were developed in order to identify potential integrins important in mediating smooth muscle cell adhesion to, contraction of and migration towards fibrin. Antibodies to $\alpha 5\beta 1$ significantly inhibited smooth muscle cell adhesion to and contraction of fibrin clots while antibodies to $\alpha \nu \beta 3$ only had a slight effect. Cell migration on fibrin was modestly affected by these antibodies; however, together both $\alpha \nu \beta 3$ and $\alpha 5\beta 1$ antibodies blocked smooth muscle migration on fibrin. A pan-integrin antagonist and disintegrin, Kistrin, also had the same effect suggesting that both integrins may play a role in smooth muscle interaction with fibrin. Because both $\alpha \nu \beta 3$ and $\alpha 5\beta 1$ are receptors for fibronectin, the role of fibronectin in smooth muscle cell interaction with fibrin was further characterized. An antibody against fibronectin inhibited smooth muscle cell adhesion and contraction of fibrin clots. Fibronectin was found on both the cell surface and in the sources of fibrinogen.

In conclusion, I have demonstrated that the integrin $\alpha 5\beta 1$ and the matrix molecule, fibronectin, play an important role in smooth muscle cell interaction with fibrin. By demonstrating that smooth muscle cells utilize these proteins to adhere, contract and migrate on a fibrin matrix, I suggest that this interaction could cause lumen narrowing during atherosclerosis. Indeed, both fibrin and fibrinogen are found in the plaque *in vivo* and

smooth muscle cells in the lesions do not express high levels of $\alpha v\beta 3$ but predominately express $\alpha 5\beta 1$. This is especially seen in areas where smooth muscle cells are co-localized with fibrin. Finally, the work presented here on the disintegrin Kistrin suggest that a pharmaceutical reagent that targets several integrins may be an effective drug to inhibit lumen narrowing during atherosclerosis progression.

Table of Contents

| | page |
|--|------|
| List of Figures | ii |
| List of Tables | iv |
| List of Abbreviations..... | v |
| Chapter 1: Introduction and Background..... | 1 |
| Chapter 2: Human Smooth Muscle Cells Interact with Fibrin..... | 21 |
| Introduction to Chapter 2 | 22 |
| Materials and Methods..... | 23 |
| Results..... | 29 |
| Discussion | 34 |
| Chapter 3: Fibronectin Plays a Critical Role in Human Smooth Muscle Cell Interaction with Fibrin..... | 49 |
| Introduction to Chapter 3 | 50 |
| Materials and Methods..... | 52 |
| Results..... | 58 |
| Discussion | 62 |
| Chapter 4: Kistrin Inhibits Human Smooth Muscle Cell Interaction with Fibrin | 78 |
| Introduction to Chapter 4 | 79 |
| Materials and Methods..... | 81 |
| Results..... | 85 |
| Discussion | 88 |
| Chapter 5: Fibrin and Integrin Expression in Human Carotid Arteries: Role of $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins in Smooth Muscle Cells Spreading and Migration..... | 97 |
| Introduction to Chapter 5 | 98 |
| Materials and Methods..... | 99 |
| Results..... | 103 |
| Discussion | 106 |
| Chapter 6: Discussion and Future Directions..... | 120 |
| Bibliography..... | 134 |

List of Figures

| | |
|--|----|
| Figure 1.1: Vessel Architecture..... | 15 |
| Figure 1.2: Staging and lesion morphology of atherosclerosis progression | 16 |
| Figure 1.3: Coagulation Cascade | 17 |
| Figure 1.4: Structure of fibrinogen..... | 18 |
| Figure 1.5: Integrin family of receptors..... | 19 |
| Figure 1.6: Inappropriate Remodeling..... | 20 |
| Figure 2.1: Integrin surface expression pattern on HNB18E6E7 and Ductus smooth muscle cells. | 39 |
| Figure 2.2: Effect of peptide antagonists and anti-integrin antibodies on plasma clot contraction by HNB18E6E7..... | 40 |
| Figure 2.3: Effect of a cyclic-peptide antagonist and anti-integrin antibodies on recombinant clot contraction by HNB18E6E7..... | 41 |
| Figure 2.4: Inhibition of cell adhesion to plasma using anti-integrin antibodies..... | 42 |
| Figure 2.5: Inhibition of cell adhesion to recombinant fibrin using anti-integrin antibodies..... | 43 |
| Figure 2.6: Effect of an anti-human vitronectin polyclonal antibody on HNB18E6E7 adhesion to plasma clots..... | 44 |
| Figure 2.7: Effect of cycloheximide on plasma clot and recombinant fibrin clot contraction by HNB18E6E7. | 45 |
| Figure 2.8: Presence of fibronectin in the supernatant and the cellular clot in the presence and absence of cycloheximide..... | 46 |
| Figure 2.9: Effect of cycloheximide on protein synthesis and clot contraction..... | 47 |
| Figure 3.1: Effect of Brefeldin A on recombinant clot contraction by HNB18E6E7..... | 67 |
| Figure 3.2: Adhesion of HNB18E6E7 smooth muscle cells to an anti-fibronectin polyclonal antibody, R790, Rabbit IgG and human fibronectin..... | 68 |
| Figure 3.3: Inhibiting cell adhesion assay using R790..... | 69 |
| Figure 3.4: Inhibiting adhesion of HNB18E6E7 smooth muscle cells to human fibronectin and collagen using R790, an anti-fibronectin polyclonal antibody | 70 |
| Figure 3.5: Effect of R790, anti-fibronectin polyclonal antibody, on whole human plasma clot contraction by HNB18E6E7..... | 71 |

| | |
|--|-----|
| Figure 3.6: Effect of R790, anti-fibronectin polyclonal antibody, on gelatin sepharose treated human plasma clot contraction by HNB18E6E7. | 72 |
| Figure 3.7: Effect of R790, anti-fibronectin polyclonal antibody, on recombinant human fibrin clot contraction by HNB18E6E7. | 73 |
| Figure 3.8: ELISA assay to detect human fibronectin in recombinant human fibrinogen and human whole plasma. | 74 |
| Figure 3.9: ELISA assay to detect fibronectin in recombinant human fibrinogen and human whole plasma. | 75 |
| Figure 3.10: Western Blot Analysis to determine the presence of fibronectin in whole plasma, gelatin sepharose treated plasma and recombinant fibrinogen. | 76 |
| Figure 4.1: Inhibition of cell adhesion to fibrin clots using anti-integrin peptides. | 92 |
| Figure 4.2: Kistrin inhibits cell adhesion to fibrin clots using. | 93 |
| Figure 4.3: Inhibiting cell adhesion to fibronectin and vitronectin. | 94 |
| Figure 4.4: Effect of integrin antagonists on fibrin clot contraction by HNB18E6E7. | 95 |
| Figure 4.5: Inhibiting cell migration on fibrin clots. | 96 |
| Figure 5.1. Cell spreading in human plasma clots. | 110 |
| Figure 5.2. Inhibition of cell spreading in plasma clots by a combination of monoclonal anti-integrin antibodies. | 111 |
| Figure 5.3A. Inhibition of cell spreading in plasma clots by cyclic RGD peptides. | 112 |
| Figure 5.3B. Invasion and migration of human smooth muscle cells into fibrin clots. | 112 |
| Figure 5.4. Western Blot Analysis using MAb #350 and MAb T2G1 on reduced carotid artery samples. | 113 |
| Figure 5.5. Specificity of MAb T2G1. | 114 |
| Figure 5.6. Expression of tissue factor and fibrin in carotid endarterectomy samples. | 115 |
| Figure 5.7. Integrin expression in diffuse intimal thickening. | 116 |
| Figure 5.8. Integrin expression in areas of intramural hemorrhage from an endarterectomy sample. | 117 |
| Figure 6.1: Model of Smooth Muscle Cell Interaction with Fibrin. | 130 |

List of Tables

| | |
|--|------------|
| Table 2.1. List of antibodies and their antigens | 48 |
| Table 3.1: Expression of fibronectin on the surface of HNB18E6E78 | 77 |
| Table 5.1. Fibrin staining of diffuse intimal thickening and plaques from coronary and carotid arteries with T2G1 | 118 |
| Table 5.2. Distribution of integrins in areas where smooth muscle cells and fibrin were both found..... | 119 |

List of Abbreviations

| | |
|---------------|--|
| ATCC | American Type Culture Collection |
| BSA | bovine serum albumin |
| CPM | counts per minute |
| DMEM | Dulbecco's Modified Eagle Media |
| ECM | extracellular matrix |
| ELISA | enzyme linked immunosorbent assay |
| FACS | fluorescence-activated cell sorting |
| FCS | fetal calf serum |
| FN | fibronectin |
| GRGDSP | glutamine-arginine-glycine-aspartic acid-serine-proline |
| GRGESP | glutamine-arginine-glycine-glutamic acid-serine-proline |
| HNB | human newborn |
| HPF | high power field |
| ICAM | intercellular adhesion molecule |
| IL | interleukin |
| mAb | monoclonal antibody |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| RGD | arginine-glycine-aspartic acid |
| RGE | arginine-glycine-glutamic acid |
| SE | standard error |
| SDS | sodium dodecyl sulphate |
| VCAM | vascular cell adhesion molecule |

Acknowledgments

I would like to thank the members of my supervisory committee, Paul Bornstein, Bill Carter, Ceci Giachelli, and Steve Hauskcha, for all of their advice throughout my graduate career. In particular I would like to express my gratitude to Bill Carter whose antibody, R790, played a crucial role in my dissertation. I especially would like to express my appreciation to my advisor, Steve Schwartz, whose support and advice has helped me develop into a better scientist. I would like to recognize all of my colleagues and friends for their invaluable moral support over the last five years. I would especially like to acknowledge members of my tennis group and members of the Schwartz, Giachelli and Reidy labs for their advice and for making the past five years a lot of fun. Finally, I would like to thank my parents, Frederick and Patricia Yee, my sister, Sharon Yee Fredericks, my brother-in-law, John Fredericks, and especially my husband, Lewis Wadsworth IV, for their unwavering support and belief in my success.

Chapter 1: Introduction and Background

Atherosclerosis is a prevalent disease that can lead to angina, stroke, plaque rupture, aneurysm or myocardial infarction and eventually cause sudden death in humans. This disease affects the large and medium size muscular and elastic blood vessels and is characterized by lesions or plaques on the vessel walls. Contrary to conventional wisdom¹, it has been observed that as atherosclerotic lesions enlarge, the vessels' lumen does not narrow. Instead, it enlarges to maintain normal blood flow through the vessel. This ability of the vessel to adapt to changes in lumen diameter has been called either adaptive remodeling or compensatory enlargement. The mechanism of adaptive remodeling is not known. It may occur as a response to factors such as the sheer stress caused by the lesion, endothelial cell factors, or medial attenuation due to the loss of structural support by proteases found in the plaque²⁻⁴.

Adaptive remodeling of atherosclerotic coronary vessels in man and animals has been studied by a number of groups. Glagov and colleagues examined the left main descending coronary arteries in hearts of patients obtained through autopsy². They found that the arteries will enlarge to maintain normal blood if the plaque takes up less than 40% of the lumen area, defined by the internal elastic lamina. If the plaque is greater than 40%, the vessel is unable to compensate for the stenosis. Clarkson and colleagues also examined the left anterior descending coronary arteries from humans as well as non-human primates³. Their results were similar to Glagov though they didn't find that 40% stenosis was a limiting factor for adaptive remodeling. Therefore, arteries are capable of adapting to the presence of a plaque. It's not clear why remodeling fails. Glagov suggested that the artery loses its ability to sense the reduction in blood flow if the plaque covers a majority of the vessel circumference (>40%). In any case, in advanced stages of atherosclerosis, the arteries will fail to compensate and the atherosclerotic plaques will eventually cause stenosis and occlusion of the vessel⁵.

Brief Historical Perspective

In the past 150 years, three main hypotheses to explain the pathogenesis of atherosclerosis have been put forth. In 1844, Rokitansky proposed that atherosclerosis was due to accumulation or encrustation of

substances at endothelial cell junctions⁶. In 1853 and 1858, Virchow suggested that atherosclerosis was the result of both an inflammatory response and an accumulation of blood lipids within the vessel wall⁷⁻⁹. In 1946 and 1948, Duguid demonstrated that thrombosis and fibrin are components of atherosclerosis progression^{10, 11}. Ross and colleagues have summarized these theories as the Response-to-Injury hypothesis and suggest that atherosclerosis is caused by injury to the endothelium by blood flow, sheer stress, chemicals and other harmful substances (i.e. tobacco smoke). This results in accumulation of lipids in the wall, a thrombotic response and plaque formation^{1, 12-14}. These processes are believed to account for the increased risk of atherosclerosis due to age, sex, race, serum lipids, dietary fat, exercise, hypertension, diabetes and cigarette smoking¹⁵.

Normal Blood Vessel Architecture

The basic architecture of the vascular wall contains three cells layers: the endothelium, the media and the adventia. The endothelium is adjacent to the lumen and is a single layer of endothelial cells that presents an anti-thrombotic surface. The media is comprised of smooth muscle cells in a basement membrane rich in extracellular matrix (ECM) components such as collagen, elastin, fibrillin, osteopontin and thrombospondin. The adventia is furthest from the lumen and is separated from the media by an elastic lamina. It is comprised of fibroblasts and small blood vessels (vaso vasorum) within a collagen matrix (Figure 1.1)^{16, 17}. Larger arteries, however, have an additional layer, the intima, which is located between the endothelium and the media and is separated from the media by a well defined layer of elastin, the internal elastic lamina. The cellular composition of the intima is complex. It includes lymphocytic and fibroblast like cells that may be derived indirectly from smooth muscle cells or blood derived precursors¹⁸.

Pathogenesis of Atherosclerosis

Atherosclerotic development is divided into five phases which are distinguished by distinct structural lesions (Figure 1.2)^{15, 19}. Phase 1 is

characterized by small plaques containing high amounts of lipids (lesions types I, II and III). Most of the lipids are in the "foam cells" which are derived from macrophage. Smooth muscle cells are present but relatively rare. Lesions of these types, however, are more widely scattered than the typical atherosclerotic lesion of the adult so it is likely that these "foam cells lesions" are fully reversible. These lesions occur during the first two decades of life and describe the majority of plaques found in the vessel. Phase 2 lesions contain a core of extracellular lipid and a fibrotic layer (type IV and Va) and appear during the third decade of life ^{15, 19}. Phase 3 atherosclerosis is marked by stenosis of the vessel by a mural thrombus, caused by rupturing of a phase 2 lesion. These complicated lesions are categorized as type VI lesions. Type VI lesions are also found in Phase 4 atherosclerosis and could cause acute coronary occlusion. Type VI lesions are complicated lesions with surface defects, hematoma-hemorrhage and thrombosis. They occur in humans starting at age 40 years. Phase 5 atherosclerosis contains type Vb and Vc lesions. Type Vb and Vc lesions contain a calcified core or a fibrotic core, respectively. They can develop from types IV or Va lesions before or after rupture of these lesions. ^{15, 19}.

According to Ross' "Reaction to Injury Hypothesis", atherosclerosis begins with endothelial cell damage¹. Damage endothelium allows the passive migration of plasma low-density lipoproteins (LDL) into the subendothelium. LDL is also transported through the endothelial cell layer via receptors. Endothelial cells as well as smooth muscle cells oxidize the LDL and trap it in the subendothelium. In response to the LDL, monocytes migrate to the subendothelium and are converted to macrophages. In an attempt to clear the oxidized LDL from the subendothelium, both the macrophages and the smooth muscle cells engulf the lipids. These lipid laden cells are known as foam cells and were described over number of years ago by Rokitansky and Virchow. These foam cells comprise the characteristic fatty streaks seen in type 1 lesions. They eventually die due to the high levels of oxidized LDL within the cells. The dead cells aid in the formation of the necrotic core found in type IV and V lesions ^{14, 20-22}.

Ross has proposed that advancement from phase 1 to phase 2 occurs when foam cells secrete growth factors such as platelet derived growth factor (PDGF), Interleukin 1(II-1), basic fibroblast growth factor (bFGF) and

macrophage-colony-stimulating factor (M-CSF). These growth factors stimulate both smooth muscle cell and macrophage proliferation. PDGF is also released by platelets adhering to the damaged endothelium and by macrophages in the area. Macrophages also secrete metalloproteinases (MMPs) and heparanases which degrade the matrix surrounding the plaque. The net result is an efflux of smooth muscle cells towards the endothelium as well as an increase in the number of smooth muscle cell derived foam cells in the lesion. Accumulation of cells and lipids will form a neointima that projects into the lumen and can cause stenosis. The smooth muscle cells will eventually form a fibrotic cap between the lipid core and the endothelial cell layer ^{14, 15, 19}.

Atherosclerosis, Thrombosis and Coagulation

At this stage in development, the atherosclerotic lesion is vulnerable to fissure and rupture due to the high lipid content, high macrophage infiltration and the small number of smooth muscle cells in the cap which inadequately supports the plaque against the stresses caused by blood flow and the rhythmic beating of the heart ²³. Grenholdt and colleagues profiled the plaques of men who died of ischaemic heart disease. They found that aortic plaques which were ulcerated or thrombosed had a high proportion (40%) of their volume occupied by extracellular lipid, had a large number of macrophages as well as a small number of smooth muscle cells. *In vivo* cell kinetic studies in several labs have consistently shown a low level of smooth muscle cell replication even in areas of what appears to be on-going injury^{24, 25}. The lack of cells in the plaque is caused by either apoptosis or cell rupture ^{21, 22} and will lead to plaque instability because less collagen, elastin and proteoglycan are deposited in the cap. This result in a softer and less stable lesion ^{15, 21, 23, 26}.

Given the prominence of the fibrous cap in early lesions, we do not know why the cap thins. This could be explained by the high density of macrophages in the ruptured plaques²⁷. Moreno *et al.* examined atherectomies from coronary arteries and observed that macrophage-rich areas were frequently found in patients who had unstable angina, and non-Q-wave myocardial infarction. The authors suggested that macrophages

could be used as a marker to identify unstable atherosclerotic plaques ²⁸. Recently, it has been shown that co-culturing macrophages with explanted fibrous caps resulted in a breakdown of the collagen in the caps, and this could be blocked by metalloproteinase inhibitors. The authors hypothesized that macrophages could release matrix-degrading metalloproteinases *in vivo* to cause destabilization of the plaque ²⁹. Similarly, Hoefstra *et al* have shown that macrophage conditioned media stimulates smooth muscle cell death by the Fas/Fas ligand pathway and this could destabilize the plaque further (Hoefstra, submitted).

A third contributor to plaque instability is calcification. During the development of advanced atherosclerosis, calcium accumulates in the core of the lesion and both macrophages and smooth muscle cells could participate in the calcification process. Calcification of the lesion could cause different properties of the plaque to be adjacent to each other, and this difference, namely at the interface between the plaque and the area of calcification, could predispose the lesion to be affected by changes in the arterial blood pressure and rupture ³⁰.

As discussed above, rupturing of the atherosclerotic plaque leads to thrombosis. Tissue factor, the activator of the coagulation cascade, is found in plaques, and upon plaque rupturing, is exposed to other coagulation proteins found in the blood ^{27, 30}. Tissue factor will bind to factor VIIa and this complex will convert Factor X to Xa ³¹. The end result of the coagulation cascade is the conversion of prothrombin to thrombin, which cleaves fibrinogen to fibrin (Figure 1.3). Platelets, activated by thrombin, will adhere to both collagen and fibrin presented in the plaque, and to the fibrin that is cleaved by thrombin. The outcome is the formation of a thrombus ¹⁷.

The thrombus may progress in several directions. One, it may be sheered from the wall by blood flow. Depending upon how large the initial plaque was, the thrombotic fragment may eventually get lodged in the vascular system and cause a stroke, infarct or mural thromboses. Two, if the plaque was small, the thrombus may be lysed by the plasminogen fibrinolytic system and eventually dissolve. Three, the thrombus will heal and organize into a type V or VI lesion. In all three cases, an open wound on the vessel wall has been healed.

Wound Healing

Surprisingly there are few studies of wound healing in the atherosclerotic wall. Most studies on wound healing focus on cutaneous wound healing^{32, 33}. A similar series of events probably occur during healing of a wound on the vessel wall. A fibrin clot first forms on the initial wound surface. Plasma proteins such as vitronectin, fibronectin and thrombospondin intercalate into the fibrin clot. Platelets adhering to the fibrin matrix release cytokines and growth factors from their granules to start the wound healing process. As the fibrin clot matures, Factor XIIIa crosslinks the fibrin strands to themselves as well as fibronectin to fibrin. Within a few days, inflammatory cells such as leukocytes and monocytes are drawn to the wound by the chemoattractants present in the fibrin clot. These cells engulf debris and bacteria that may be present in the wound. Monocytes, which are converted to macrophages *in situ*, will also release a number growth factors and cytokines in the wound area that are important for healing³³.

Reepithelization of cutaneous wound occurs in two directions. Keratinocytes in the basal lamina of the wound will start to proliferate and migrate towards the provisional matrix provided by the fibrin clot. At the same time, these keratinocytes express proteases, such as tissue-type plasminogen activator (tPA), urokinase-type plasminogen activators (uPA) or metalloproteinases (MMPs), to dissolve the fibrin matrix and facilitate migration. tPA and uPA cleave plasminogen to plasmin which in turn degrades fibrin. Members of the MMP family dissolve extracellular matrices such as collagen and laminin. The second area of reepithelization occurs at the dermal surface of the wound. Fibroblasts at the wound edge proliferate and migrate into the provisional matrix (fibrin clot) in response to the growth factors present. They will also start to lay down a collagen rich matrix, a key component of granulation tissue³³. With the proper balance of connecting tissues, cells are able to migrate, proliferate and synthesize matrix proteins and enhance wound healing rather than lyse wounds as we believe happens in the advanced plaque. Thus, it is likely that plaque rupture depends, in part, on the failure of the smooth muscle cells in advanced lesions to repair the wound.

Returning to the skin as a model of wound healing, granulation tissue appears around Day 4 after wound formation. The characteristic pink color of granulation tissue is due to the presence of a vast number of blood vessels that feed the healing tissue. McClain and colleagues studied cutaneous wound healing in pigs to determine why there exists a lag time in granulation tissue formation³⁴. They found that the combination of fibrin, fibroblast and platelet releasates must to be present in order to activate mesenchymal cells to induce granulation tissue formation. To the extent cutaneous wound healing is understood today, the rate-limiting factor would be the activation and migration of fibroblasts from the dermal surface into the fibrin clot.

Pathological Wound Contraction in Atherosclerosis: A Hypothesis

The last phase of healing is closure of the wound edges. Over time, reepithelization will decrease the wound area. Once tension on the wound edges reach a certain strength, fibroblasts, activated by growth factors such as TGF- β , will be transformed into myofibroblasts, smooth muscle cell α -actin expressing cells. Closure of the wound is caused by myofibroblast migration. As the myofibroblast migrate, they pull the edges of the wound together. Once the edges meet, tension on the wound edges is relaxed, and fibroblasts cease to migrate and become quiescent. Healing is complete upon the closure of the wound though several months may pass before any scarring is resolved^{32, 35}.

It is tempting to think that a similar process might narrow the lumen of a vessel following rupture. Although the Response to Injury hypothesis posited that the accumulation of cells, matrix molecules and lipids on the vessel wall is responsible for the narrowing of the lumen in atherosclerosis, as we have stated, narrowing does not occur until lesions become very advanced¹. However, if the plaque ruptures and thrombosis occurs, then the narrowing of the lumen could occur as a normal outcome of wound healing. In contrast, failure to heal might explain unstable angina. Pathological consequences of contraction have been seen during healing of severe wounds such as burns and in hollow organs such as esophagus³⁶.

Recently, we have demonstrated that pathological contraction could be responsible for vessel narrowing after vascular injury. Courtman *et al.* combined a double balloon injury rabbit model with a factor VIIa inhibitor, called DEGR-VII³⁷. By inhibiting binding of factor VIIa to tissue factor, the generation of thrombin was prevented. Their studies demonstrated that control rabbits, which were not infused with DEGR-VII during the second balloon injury of the abdominal aorta, developed a smaller lumen 3 months after the second injury as compared with treated rabbits. Furthermore, maintenance of lumen was associated with less fibrin deposition on and in the vessel of the treated animals as compared with the control. Importantly, the areas of the media and intima in the two groups did not differ, suggesting that the difference in lumen size was not due to mass but to contraction or remodeling. This study also suggests that fibrin may play a role in vessel narrowing after vascular injury.

The cell generating the contractile force and responsible for lumen narrowing is presumably the intimal smooth muscle cells. Courtman *et al.* showed that fibrin formation was restricted to the intima. Smooth muscle cells are the only cell type in the intima at three months after injury and would have the ability to contract. Furthermore, the myofibroblasts that are found in cutaneous wounds and responsible for wound closure are very similar to smooth muscle cells. Gabbiani and colleagues first identified the existence of myofibroblasts in wounds³⁸. They found that fibroblasts in granulation tissue were very similar to smooth muscle cells by electron microscopy and immunofluorescent. They demonstrated that strips of granulation tissue as well as blood clots from rats are able to contract when exposed to a number of pharmacological agents that have been shown to promote smooth muscle cell contraction. Using anti-serum against human smooth muscle cells, they identified the presence of myofibroblasts in granulation tissue at day 9, 11 and up to day 15 of wound healing. In the atherosclerotic plaque, smooth muscle cells can take the place of myofibroblasts and fulfill a similar role. However, in the vasculature the contraction that occurs during wound healing could be pathological in nature.

Geary *et al* looked at wound healing after vascular injury of pre-existing atherosclerotic lesions in the iliac arteries of non-primate

monkeys³⁹. These monkeys were fed an atherogenic diet for several years and then underwent vascular injury using a balloon catheter. This procedure, called percutaneous transluminal angioplasty, is used in humans to unblock stenotic lesions mechanically. The authors followed the healing process up to 28 days post injury. They found a pattern very similar to cutaneous wound healing with the majority of contractile cells being smooth muscle α -actin positive cells (smooth muscle cells/myofibroblasts). They concluded that because wound healing in the arteries was so similar to cutaneous wound healing, contraction as a mechanism of lumen narrowing during atherosclerotic progression was plausible.

Fibrinogen and Fibrin

Courtman and colleagues' study implicated fibrinogen or fibrin as a key component in lumen narrowing. Fibrinogen is a symmetric disulfide-linked homodimer, and each monomer contains 3 polypeptide chains ($A\alpha$, $B\beta$ and γ) (Figure 1.4). Fibrinogen is converted to fibrin via thrombin, a serine protease, which cleaves the A and B fragments off the α and β chains, respectively. Cleavage creates *de novo* binding sites which allow fibrin homodimers to anneal to form a profibril⁴⁰. The monomers are further crosslinked together by Factor XIIIa, which is also activated by thrombin⁴¹. There are two RGD cell recognition motifs found on the $A\alpha$ chains (residues 95-98 and 572-575) of fibrinogen, and they may serve as cell adhesion sites. On the γ chain there is a third binding site, the dodecapeptide (HHLGGAKQAGDV) region (residues 400-411)^{42, 43}.

Fibrinogen has been found to be a normal component of the vessel wall. Several groups have found the protein in the intima layer of the wall even without signs of thromboses occurring⁴⁴⁻⁴⁷. Retention of fibrinogen in the wall may depend on crosslinking by tissue transglutaminase rather than being converted to fibrin⁴⁸. Fibrin is also found in the wall and usually confined to the atherosclerotic plaque. There is evidence that this fibrin is formed in situ by tissue factor trapped in the plaque^{49, 50}. Levels of fibrinogen has been suggested to be predictive of cardiovascular disease state and has been associated with coronary artery disease, carotid disease and peripheral arterial disease⁵¹. Fibrinolysis could reduce plaque size;

however, the fibrin degradative products have been shown to be mitogenic^{50, 52, 53}. Fibrinogen and fibrin can also serve as a reservoir for many proteins. Lp(a) and fibronectin have been shown to bind to fibrin and they are both found in plaque^{46, 53-55}. Recently, one group has shown biochemically that FGF also binds fibrin⁵⁶.

Smooth Muscle Cell-Fibrin Interaction

Nucleated cells, such as smooth muscle cells, as well as platelets and leukocytes bind to the ECM through a family of receptors called integrins. Integrins are heterodimeric surface receptors composed of a non-covalently linked α and β chain. In general, integrins have a large, globular extracellular domain (NH₂) and a short cytoplasmic tail (COOH), and function in cell-ECM interactions (Figure 1.5)^{57, 58}. In most cases, integrins bind to an Arginine-Glycine-Aspartic Acid (RGD) motif in the ligand. To date, 16 α chains and 7 β chains have been identified, and their ligands include collagen type I and IV, fibronectin (FN), laminin (LM), fibrinogen/fibrin, osteopontin (OPN), thrombospondin (TSP), vitronectin (VN), Von Willebrand Factor (vWF) and ICAM 1, 2 and 3⁵⁸.

Only four integrins have been shown to bind to fibrinogen: α M β 2, α IIb β 3, α 5 β 1 and α v β 3. α M β 2 is expressed in leukocytes, α IIb β 3 is only expressed in platelets and α v β 3 and α 5 β 1 are expressed on a variety of cell types including smooth muscle cell, endothelial cells, and melanoma cells⁵⁷. Synthetic peptides from the N termini of the β 3 subunit can prevent binding of purified α IIb β 3 to fibrinogen⁵⁹. Also in cross-linking experiments, RGD-peptides have been reported to bind to β 3 fragments⁶⁰. It has been shown that the dodecapeptide region of fibrinogen binds to the α IIb β 3 integrin in both crosslinking experiments and inhibition experiments using synthetic peptides^{61 62}. Furthermore, there are plasma proteins such as TSP, FN and vWF which can bind the A α chain of fibrinogen as well as interact with integrins other than α v β 3 such as α 3 β 1, α 4 β 1 and α 5 β 1^{40 63}. Since these molecules use additional integrins to adhere to cells, they may act as bridge molecules and increase the repertoire of integrins that can interact with fibrinogen.

Immunohistochemistry has been used to identify the integrins present in the vessel wall with a focus on the integrins that have been implicated by *in vitro* studies as playing key roles in smooth muscle cell migration. These include $\alpha 2\beta 1$, a collagen receptor⁶⁴, $\alpha v\beta 3$, a fibrin and denatured collagen receptor⁶⁵, and $\alpha 5\beta 1$, a fibronectin and fibrinogen receptor⁶⁶. Despite some excellent studies *in vitro*, *in vivo* studies have only shown $\alpha 2\beta 1$ in the endothelium⁶⁵. In contrast, both fibrin integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ are abundant in the vascular media, as shown by Hoshiga *et al*⁶⁵. $\alpha 5\beta 1$ is also found in most plaque smooth muscle cells as well as in the media⁶⁶. $\alpha v\beta 3$ is abundant in carotid smooth muscle cells but only can be found in a subset of intimal or plaque smooth muscle cells⁶⁶. Thus, *in vivo*, integrins that are receptors for fibrin are expressed by human smooth muscle cells *in vitro* and *in vivo*.

In Vitro Models to Study Wound Contraction

To elucidate the mechanism of wound contraction, *in vitro* models such as collagen-fibroblast or fibrin-fibroblast matrices have been used to observe cell-matrix interactions. The integrin that has been implicated in the interaction between fibroblasts or smooth muscle cells with collagen is $\alpha 2\beta 1$ ³². Unfortunately, as noted earlier, the expression of $\alpha 2\beta 1$ on smooth muscle cells is an *in vitro* artifact⁶⁴. Studies using fibrin matrices have not been as extensive as collagen, and most of the work has involved fibrin and platelet interactions. However, a number of studies have demonstrated that the integrin $\alpha v\beta 3$ is important for fibrin clot contraction by nucleated cells. Ylanne *et al.* demonstrated that chinese hamster ovary (CHO) cells, nucleated cells which have been transfected with the human $\alpha IIb\beta 3$ integrin, are able to contract fibrin clots *in vitro*, and this event is dependent on the $\beta 3$ subunit⁶⁷. In a recent paper from the same group, they showed that this contraction is mediated by $\alpha v\beta 3$ where the integrin is composed of the hamster αv subunit and the human $\beta 3$ subunit⁶⁸. Furthermore, Kitagiri *et al.* have shown that melanoma cells, which express αv , are able to contract fibrin clots after being transfected with the $\beta 3$ subunit⁶⁹.

Thesis Studies

Due to the evidence that $\alpha v\beta 3$ integrin binds fibrinogen/fibrin, is expressed on smooth muscle cell *in vivo*, and has been shown to mediate fibrin gel contraction by nucleated cells, we originally hypothesized that $\alpha v\beta 3$ may be one of the integrins mediating smooth muscle cell and fibrin interactions during atherosclerosis. This hypothesis has turned out not to be entirely correct. As already noted, studies of smooth muscle cell interaction with collagen *in vitro* are of little value *in vivo* because this integrin appears to be an *in vitro* artifact. Fortunately for us, $\alpha 2\beta 1$ is not relevant to smooth muscle cell interaction with fibrin⁷⁰.

In the following chapters, I will describe the integrins that are important in smooth muscle cell adhesion to, contraction of and migration on fibrin. These interactions will be determined using a number of antibodies, peptides and chemical reagents. I have been especially concerned about the choice of cell lines. Two smooth muscle cells strains, HNB18E6 E6E7 and Ductus smooth muscle cells, will be used in the work presented in Chapter 2. Only HNB18E6E7 will be used in the subsequent chapters. These cultured smooth muscle cells display several characteristics of intimal smooth muscle cells which are found in the plaque. These smooth muscle cells are not quiescent and are actively synthesizing a number of extracellular matrices^{71,72}. As I will show, they express $\alpha v\beta 3$, $\alpha 5\beta 1$ and $\beta 1$ integrins. Therefore I believe that these cultured smooth muscle cells adequately represent smooth muscle cells found in an atherosclerotic plaque.

Three sources of fibrinogen will also be used in all three assays. These differ in the levels of other plasma proteins. The simplest clots are made from human recombinant fibrinogen that is expressed in chinese hamster ovary cells. This should not contain any other plasma proteins besides human fibrinogen. Gelatin sepharose treated human plasma will also be used. This preparation is frequently employed to eliminate fibronectin from the studies of fibrin clot contraction⁶⁷. This source of plasma does contain the other plasma proteins normally found in plasma, such as vitronectin. My third source of fibrinogen is human plasma which contains all the protein normally found in plasma and represents the *in vivo* milieu of a fibrin clot.

The work presented here extends the body of knowledge regarding cell interaction with fibrin to include human smooth muscle cell adhesion, contraction and migration on fibrin. By demonstrating that smooth muscle cells are capable of interacting with a fibrin clot, specifically contracting a fibrin clot, the hypothesis that smooth muscle cell mediated fibrin clot contraction as a mechanism of lumen narrowing in atherosclerosis is feasible (Figure 1.6). This work also presents a novel target, $\alpha 5\beta 1$, and a bridge protein, fibronectin, as possible targets to be used in pharmaceutical intervention to inhibit atherosclerosis progression.

Arterial Architecture

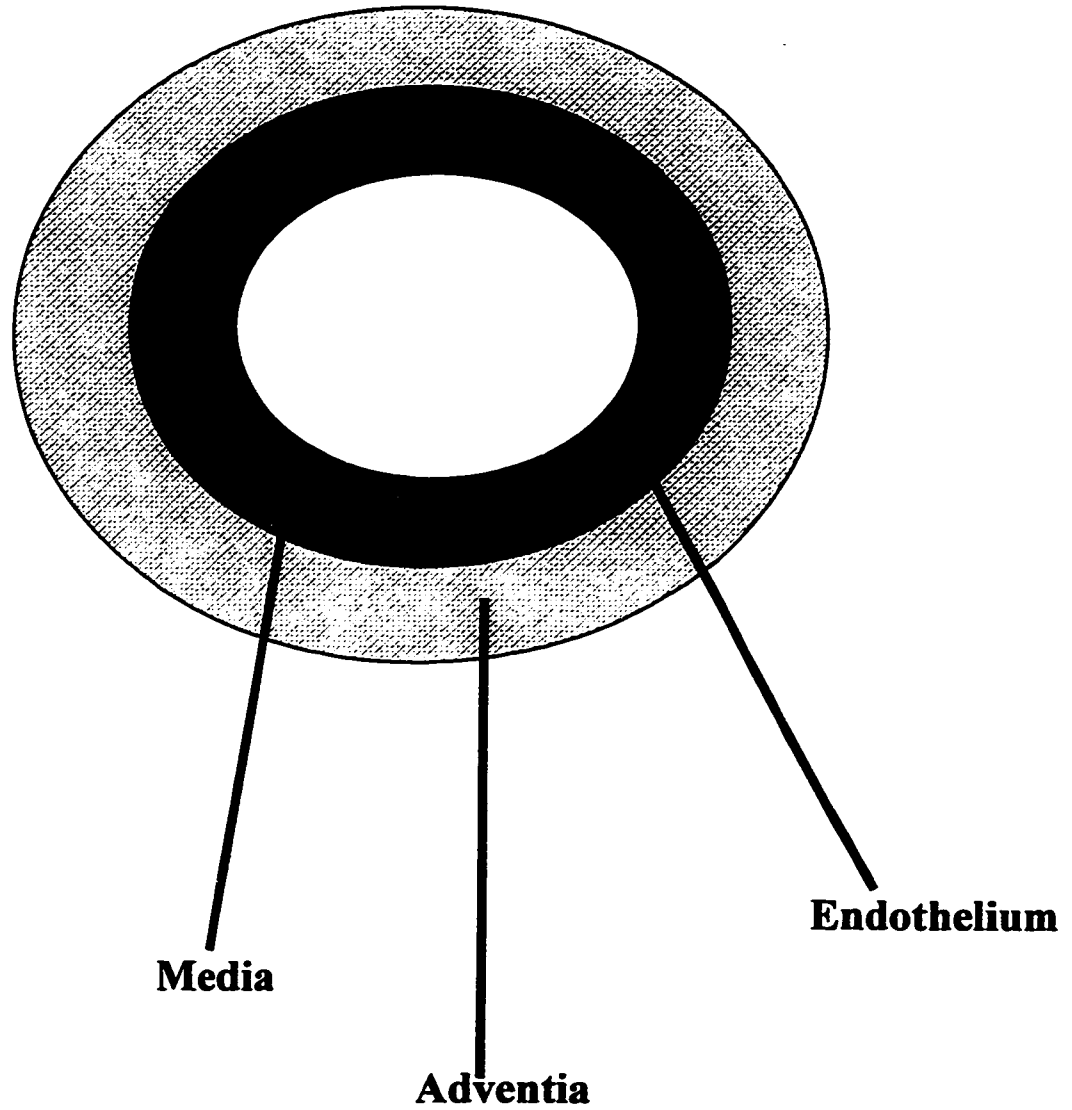


Figure 1.1: Vessel Architecture

**Staging and Lesion Morphology of
Atherosclerosis Progression
(Stary *et al.*, 1995)**

| <u>Phase</u> | <u>Lesion Types</u> | <u>Description</u> |
|---------------------|----------------------------|--|
| 1 | I-III | macrophages, lipid droplets, SMC |
| 2 | IV-Va | extracellular lipid, lipid core, collagen, SMC cap |
| 3 | VI | disruption of plaque and thrombus; mural |
| 4 | VI | occlusion and reocclusion |
| 5 | Vb | collagen, SMC layer with lipids, calcification |
| | Vc | same as Vb but has a fibrotic core |

Figure 1.2: Staging and lesion morphology of atherosclerosis progression

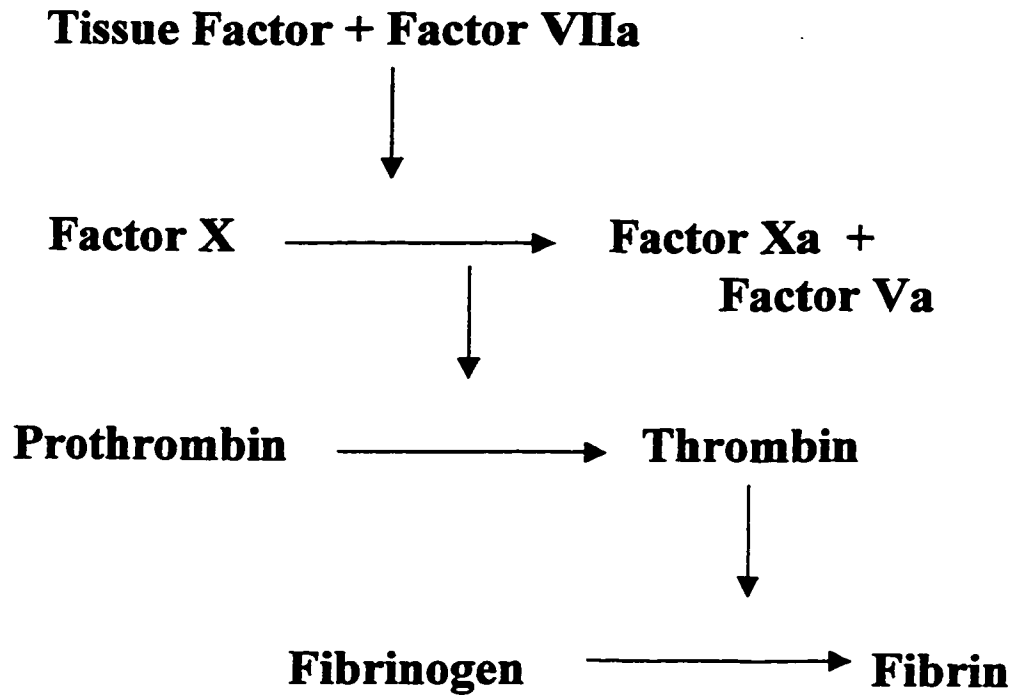


Figure 1.3: Coagulation Cascade

Fibrinogen Monomer

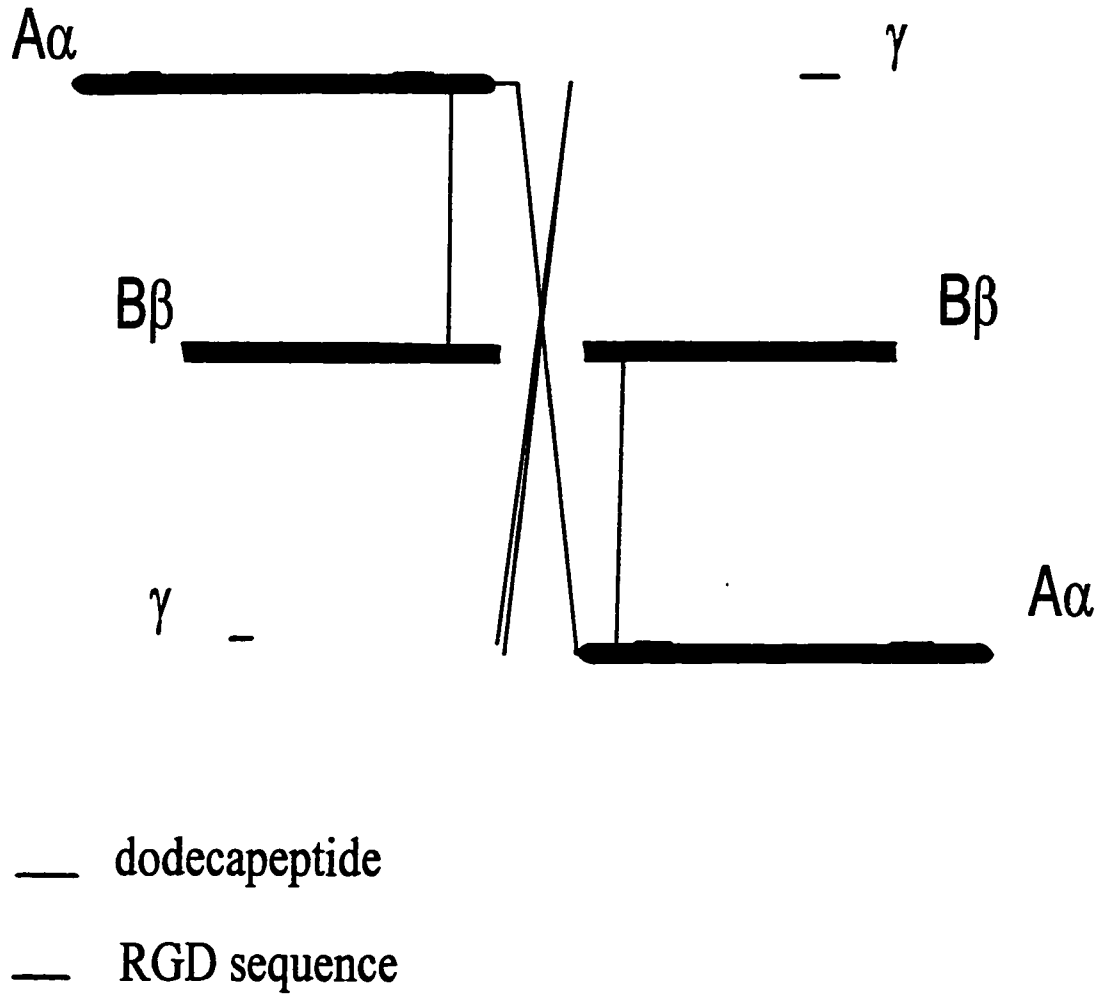


Figure 1.4: Structure of fibrinogen

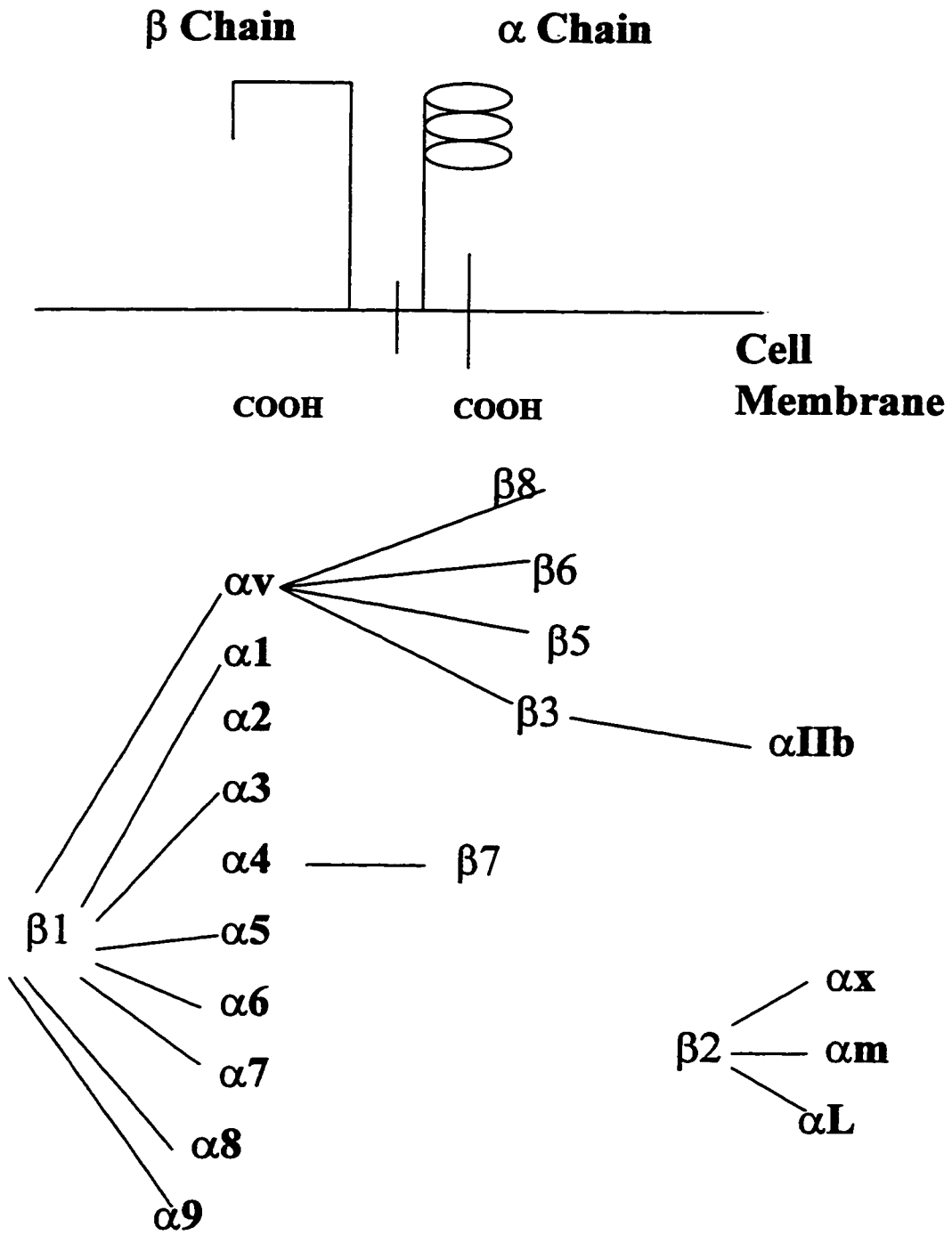


Figure 1.5: Integrin family of receptors

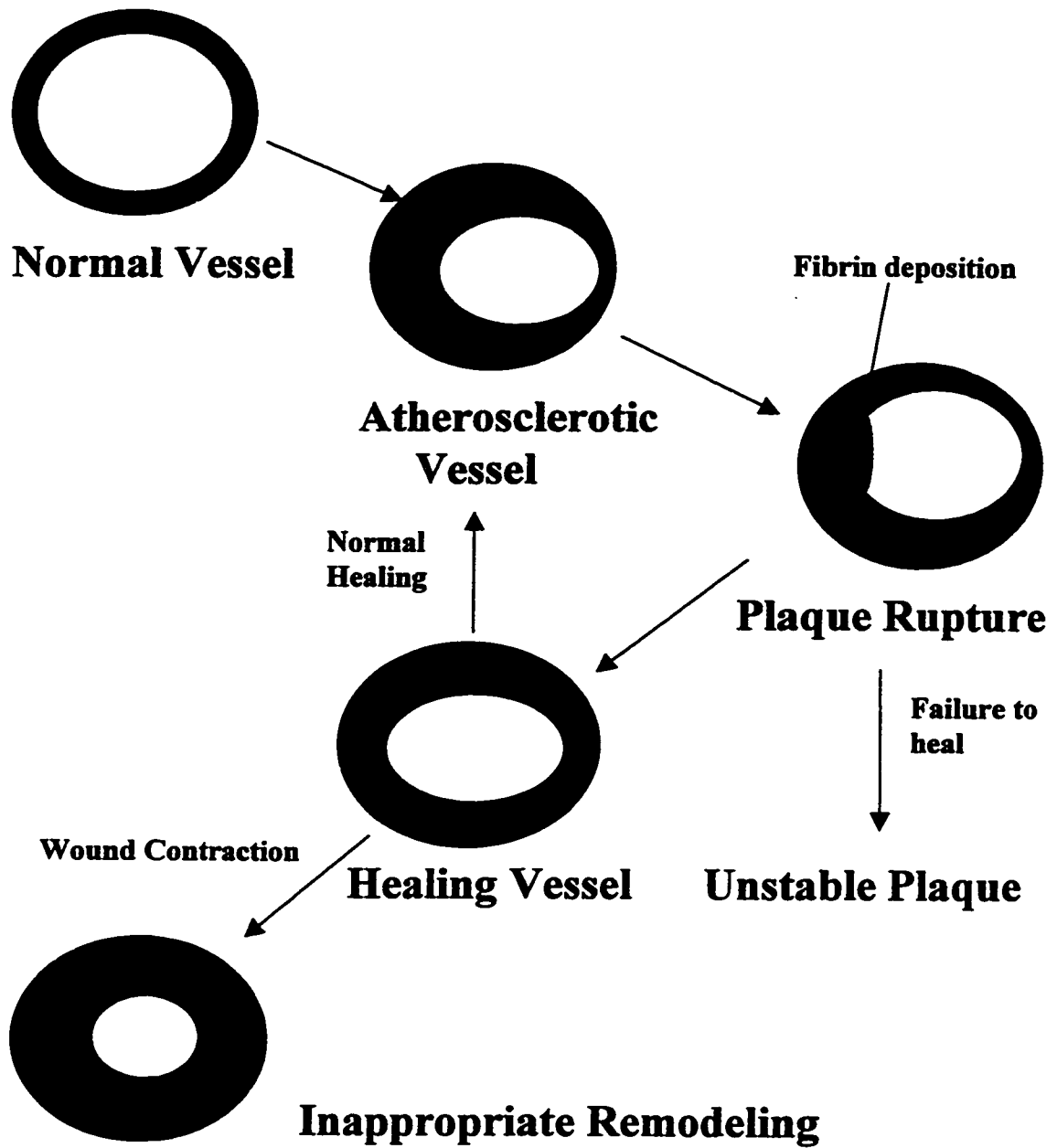


Figure 1.6: Inappropriate Remodeling

Chapter 2: Human Smooth Muscle Cells Interact with Fibrin

This work was published in similar form by Karen O Yee, Michael M. Rooney, Cecilia M. Giachelli, Susan T. Lord and Stephen M. Schwartz in *Circulation Research*, 1998. Vol 83, No. 3, pp. 241-251.

Introduction to Chapter 2

It is generally believed that the accumulation of cells, matrix molecules and lipids on the vessel wall is responsible for the gradual narrowing of the lumen in atherosclerosis.¹ Recent studies, however, have shown that lumen size correlates poorly with atherosclerotic lesion mass in either primary or restenotic vessels.^{2, 3, 73, 74} These studies demonstrate that arteries enlarge at lesion sites and thereby maintain normal blood flow. This ability of the vessel to adapt to changes in lumen diameter has been called compensatory enlargement or remodeling.^{2, 3} Thus, it is reasonable to hypothesize that the pathologic narrowing of the lumen in atherosclerosis may arise from a failure of remodeling or an active narrowing at certain lesion sites.

We have considered the possibility that pathologic narrowing results from plaque rupture, a process characteristic of advanced lesions.^{26, 27, 30, 75} When plaques rupture, platelets adhere to exposed collagen. Next, tissue factor is released, initiating the coagulation cascade. Activation of thrombin leads to formation of fibrin.^{17, 19, 31, 50} By analogy to other wounds, the thrombus situated at the ruptured lesion site could serve as a focus for subsequent healing and presumably wound contraction.⁷⁶ Thus the role of fibrin-smooth muscle cell interactions could be critical to vascular narrowing in atherosclerosis.

It is important to note that unlike other types of wounds, the atherosclerotic plaque is chronic and characterized by the persistent presence of fibrin, fibrinogen and fibrin degradative products in the vessel wall.^{17, 19, 26} Because active thrombin is found in plaques, it is reasonable that plaque fibrinogen is converted to fibrin *in situ*.⁵⁰ Fibrin is present not only in thrombi associated with acutely ruptured plaques, but also at sites of chronic injury.^{46, 47} Crosslinked fibrinogen is also abundant in the intimas of atherosclerotic aortas.^{44, 48}

There is data indicating that fibrin provides a scaffold for smooth muscle cell migration.^{26, 46} Cells interact with fibrin via integrins, which are heterodimeric surface receptors composed of a non-covalently linked α and β chain.^{57, 58} Three integrins have been shown to bind to fibrin: α M β 2, which is expressed on leukocytes, α IIb β 3, which is expressed on platelets and

megakaryocytes, and $\alpha v\beta 3$, which is expressed on a variety of cells including smooth muscle, endothelial and melanoma cells.^{57, 77-80} Recently, three groups have reported that cells can also adhere to fibrinogen via a $\beta 1$ integrin. Marshall *et al.* demonstrated that adhesion of human melanoma cells to fibronectin depleted fibrinogen is inhibited by antibodies against αv and $\beta 1$.⁸¹ Neugebauer *et al.* showed that adhesion of a chicken myeloblast cell line to commercial fibrinogen was inhibited by an anti- $\alpha v\beta 3$ antibody and an anti- $\beta 1$ antibody.⁸² Suehiro *et al.* implicated $\alpha 5\beta 1$ as a receptor for fibrinogen on endothelial cells in the presence of manganese.⁸³

Similar to platelets, nucleated cells can contract a fibrin clot *in vitro*. Early reports demonstrated that bovine endothelial cells and smooth muscle cells can contract a fibrin clot.⁸⁴ More recently, cell mediated fibrin clot contraction has been demonstrated with human melanoma cells, which express $\alpha v\beta 3$, and transfected Chinese hamster ovary cells (CHO) that express human $\beta 3$ complexed with hamster αv .⁶⁷⁻⁶⁹

The studies described here examine whether human smooth muscle cell lines are able to contract fibrin clots composed of either gelatin sepharose treated plasma ("plasma clots") or recombinant fibrinogen ("recombinant fibrin clots"). Using integrin specific antibodies, as well as specific peptide inhibitors, we investigated which integrins were utilized by the smooth muscle cell lines to contract and adhere to fibrin. We report that contraction of fibrin clots by cultured smooth muscle cell lines was not inhibited by antibodies to $\alpha v\beta 3$ but by antibodies to $\alpha 5$, $\beta 1$ and $\alpha 5\beta 1$. We also found that smooth muscle cell adhesion to plasma clots differed from adhesion to recombinant fibrin. These data suggest that smooth muscle cell contraction of fibrin clots involves the $\alpha 5\beta 1$ integrin, and smooth muscle cell adhesion to plasma clots involves at least the participation of one plasma protein, vitronectin, and possibly others. The fact that the smooth muscle cells are able to contract a fibrin matrix *in vitro* suggest that *in vivo*, they could do the same and cause vessel narrowing.

Materials and Methods

Flow Cytometry

Human smooth muscle cell lines were harvested with trypsin-EDTA (GIBCO/BRL; Gaithersburg, MD), washed twice in PBS containing 0.5 mg/ml of Soybean Trypsin Inhibitor (Sigma Chemical Co; St. Louis, MO) and incubated on ice with the primary antibody (1:100 dilution) in 50 μ l of PBS containing 0.2% BSA (Sigma Chemical Co; St. Louis, MO) for 2 hours. The samples were subsequently washed three times with PBS containing 0.2% BSA, and incubated for 1 hour with a phycoerythrin conjugated goat anti-mouse IgG secondary antibody (Biomedica; Foster City, CA) at a concentration of 0.2 mg/ml in PBS containing 0.2% BSA. Cells were washed in PBS and fixed using 1% paraformaldehyde. Peak Fluorescence Intensity was measured on a FACScan flow cytometer (Becton Dickinson Immunocytometry System; San Jose, CA).

Antibodies and Peptides

Mab 1976/LM609 (anti-human integrin α v β 3),⁸⁵ Ascites P4G9 (anti-human integrin α 4),⁸⁶ Mab 1980 (anti-human integrin α v),⁸⁷ Ascites JBS5 (anti-human integrin α 5 β 1)⁸⁸ and Ascites LM534 (anti-human integrin β 1)⁸⁹ were purchased from Chemicon International (Temecula, CA). Ascites P4C10 (anti-human integrin β 1),⁹⁰ Ascites P1E6 (anti-human integrin α 2),⁹¹ Ascites PIF6 (anti-human integrin α v β 5),⁹² Mab P1B5 (anti-human integrin α 3),⁹³ Peptide GRGDSP, Peptide GRGESP, penRGD⁹⁴ and Ascites Clone I (anti-human fibronectin)⁹⁵ were purchased from GIBCO/BRL (Gaithersburg, MD). Mab 16 (anti-human integrin α 5) was purchased from Becton Dickinson (San Jose, CA).^{96, 97} Mouse IgG was purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). c7E3 Fab Fragment Mab (anti-human integrin α v β 3 and α IIb β 3) was a gift from Drs. Robert Jordan and Jim Woody at Centocor (Malvern, PA).^{68, 98-100} Anti-human integrin α 1 Mab (5E8D9)¹⁰¹ and Mab DE9 (anti-human integrin β 1)¹⁰² were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal antibody Y9A2 (anti-human integrin α 9)¹⁰³ was a gift from Dr. Dean Sheppard. Monoclonal antibody JIB5, anti-human integrin α 6, was provided by Dr. Caroline Damsky.¹⁰⁴ Anti-human vitronectin polyclonal antibody was a gift from Dr. Klaus T. Preissner.¹⁰⁵

Purification of L230 Hybridoma Supernatant

The hybridoma cell line L230 was purchased from ATCC (Rockville, MD). This antibody blocks all αv integrins.¹⁰⁶ The cell line was cultured in RPMI 1640 media (BioWhittaker; Walkersville, MD) supplemented with 0.35 mg/ml L-glutamine, 1 mmol/L sodium pyruvate, and 0.01 mmol/L non-essential amino acids which were all purchased from GIBCO/BRL (Gaithersburg, MD). To this media 10% FCS (HyClone Laboratories, Inc.; Logan, UT) was added. The cells were allowed to grow to confluence and the media was harvested by spinning at 1000 x g for 10 minutes and filtering through a 0.45 μ m filter. The media, diluted 1:1 with Pierce ImmunoPure Gentle Ag/Ab binding buffer (Rockford, IL), was placed on a goat-anti-mouse IgG agarose column (Sigma Co; St. Louis, MO) that was previously equilibrated with Pierce ImmunoPure Gentle Ag/Ab binding buffer (Rockford, IL) at room temperature. The column was washed with binding buffer and the flow-through was saved to be reloaded on the column. The antibody was eluted using Pierce ImmunoPure Gentle Ag/Ab Elution Buffer (Rockford, IL), and dialyzed against Tris buffer (50mmol/L Tris, pH 7.5 and 100mmol/L NaCl) overnight at room temperature. The antibody was concentrated using Centriprep 10 Concentrators (Amicon, Beverly, MA).

Purification of P4C10 Ascites

P4C10, purchased from GIBCO/BRL (Gaithersburg, MD) was resuspended in binding buffer (0.01 mol/L NaPO₄, pH 7 and 0.015 mol/L NaCl) and loaded onto a recombinant protein G Agarose column that was previously equilibrated with binding buffer at 4°C. The IgG antibody was eluted with 0.1mol/L glycine hydrochloride, pH 2.6, at 4°C. The eluted antibody was brought to pH 7 using 1mol/L Tris, pH 8, dialyzed against PBS overnight at 4°C and concentrated using Centricon 10 Concentrators (Amicon, Beverly, MA).

Cell Culture

Human Newborn 18 smooth muscle strain has been described previously and was originally derived from the aorta of a newborn infant (2-day old) autopsy.¹⁰⁷ This strain has been transfected with a plasmid containing the type 16 human papilloma virus proteins E6 and E7, which

interact with p53 and Rb and cause immortalization of the cell.¹⁰⁸ Thus, the strain is now referred to as Human Newborn 18 E6E7 (HNB18E6E7). E361812 (ductus smooth muscle cell) is a non-immortalized smooth muscle strain derived from the region of the aorta adjacent to the ductus arteriosus from a human newborn. This strain was a gift from J. Slomp (Rijks University, The Netherlands). By immunocytochemistry, these cells express α -actin, a marker for smooth muscle cells (unpublished data, 1998). Both strains were grown in Waymouth media (GIBCO/BRL; Gaithersburg, MD) containing 0.23% sodium bicarbonate, 0.35 mg/ml L-glutamine, 1 mmol/L sodium pyruvate, and 0.01 mmol/L non-essential amino acids (GIBCO/BRL; Gaithersburg, MD). This basic media was supplemented with 10% FCS (HyClone Laboratories, Inc.; Logan, UT). HNB18E6E7 smooth muscle cells were harvested at passages 24-30, and Ductus smooth muscle cells were harvested at passages 15-20.

Plasma, Recombinant Fibrinogen and Thrombin

Citrated human blood was centrifuged at 150 x g for 15 minutes at 20°C and the subsequent platelet-rich plasma was then centrifuged at 1400 x g for 20 minutes at 20°C. The platelet-poor plasma was placed on a gelatin-sepharose 4B column (Pharmacia; Uppsala, Sweden) that was previously equilibrated with a buffer containing 0.05 mol/L Tris-HCL, pH 7.5, 5 mmol/L benzamidine and 0.02% sodium azide. The gelatin sepharose treated plasma was collected and the extent of fibronectin depletion determined by Western Blot analysis.^{109, 110} In all cases, 80-90% of the fibronectin was removed from the plasma. The gelatin sepharose treated plasma was stored at -70°C. In the text, plasma will refer to gelatin sepharose treated plasma. The production, purification and characterization of recombinant fibrinogen has already been described.^{111, 112} Human α -thrombin was purchased from Sigma Chemical Co. (St. Louis, MO).

Adhesion Assay

25 μ l of plasma, diluted 1:5 with PBS, was added to each well of a 96 well immunosorbant plastic plate (Nunc; Naperville, IL). An equal amount of thrombin (5units/ml) was then added to each well and the plate was stored overnight at 4°C. The wells were washed twice with PBS, blocked

with 10 mg/ml of BSA-PBS for 1 hour at 37°C and in some experiments, pre-incubated with an anti-human vitronectin polyclonal antibody for 15 minutes. The smooth muscle cells (50,000 cells/well) were pre-incubated for 15 minutes at room temperature with the indicated antibody in serum-free Waymouth media containing 1 mg/ml of BSA, and then allowed to adhere to the substrate at 37°C/5% CO₂ for 1 hour. Non-adhering cells were washed off using PBS that was pre-warmed at 37°C, and the adhering cells were fixed in 4% paraformaldehyde and stained with 4% paraformaldehyde containing 0.5% toluidine blue. The color was solubilized using 1% SDS and color intensity was measured using an ELISA reader set at 595nm¹¹³. All experiments were carried out independently at least twice with n=3 for each group.

Plasma Clot Contraction Assay

This assay is based on the one published by Ylanne *et al.*³¹ Cultured human smooth muscle cell lines were harvested using trypsin-EDTA, washed twice in 0.5mg/ml of soybean trypsin inhibitor (Sigma Chemical Co.; St. Louis, MO), and resuspended at a concentration of 10⁷ cells/ml in serum-free, Hepes (25mmol/L) buffered-DMEM (GIBCO/BRL; Gaithersburg, MD). 40µl of diluted human gelatin sepharose treated plasma was added to 700,000 smooth muscle cells (70µl) in a 6mm X 50mm pre-siliconized, autoclaved borosilicate test tube. For the HNB18E6E7 smooth muscle cells, the optimal plasma: Hepes-DMEM ratio was 1:5 while for the ductus smooth muscle cell, it was 1:3. Lastly, 40µl of a mixture containing 28mmol/L CaCl₂ and 5 units/ml of thrombin in serum-free, Hepes-DMEM was added to the test tube. The test tubes were incubated at 37°C with 5% CO₂. Pictures were taken with an Olympus OM2 camera using Kodak Ektachrome T64 slide film (Rochester, NY) and a Zuiko 50mm f3.5 macro lens at t=0 and at time points specified in the figure legends. The slide was projected onto a piece of paper and the clot was traced. From the tracing, a two dimensional area was calculated to determine the percent of clot contraction using the following equation.

$$\% \text{ Clot Contraction (t= 8 hrs)} = 100 - \frac{[\text{area t=8 hrs} \times 100]}{\text{area at t=0}}$$

The mean and the standard error (SE) were calculated (Microsoft Excel; Redmond, WA). All experiments were carried out independently at least twice with $n=3$ for each group. One representative experiment is shown.

Recombinant Fibrin Clot Contraction Assay and Adhesion

The assays were similar to what was described above except that recombinant fibrinogen^{111, 112} at a final concentration of 0.2mg/ml, was used instead of human gelatin sepharose treated plasma.

Metabolic Labeling with Cycloheximide

Equal numbers of human smooth muscle cells were plated on 60mm tissue culture dishes and grown to 70% confluence. At that time they were washed once with PBS, once with methionine free DME media (Gibco/BRL; Gaithersburg, MD) and incubated for 30 minutes at 37°C in methionine free DME media. Methionine free DME media containing ³⁵S-methionine (Amersham Life Science, Inc.; Arlington Heights, IL) with or without cycloheximide (80µg/ml) was added to the cells and the cells were incubated at 37°C. At the time points listed on the graph, the cells were washed twice with PBS and lysed on ice using an extraction buffer (PBS, 1% Triton X-100) containing a protease inhibitor cocktail (Pierce; Rockford, IL). After 10 minute, the lysates were scraped from the plate and 400 µl was placed in EcoLite scintillation cocktail (ICN; Costa Mesa, CA). The amount of radioactivity was measured using a Beckman LS 5000CE scintillation counter (Palo Alto, CA).

Western Blot Analysis of Fibronectin proteins

2X SDS sample buffer was added to either the supernatant or the cells obtained from the contraction assay. The proteins were electrophoresed on a 5% SDS polyacrylamide gel and then transferred to PolyScreen PVDF transfer membrane (NEN Research Products; Boston, MA) using standard protein transfer techniques. The PVDF membrane was first probed with an antibody against human fibronectin at 1/100,000 dilution followed by a goat anti-mouse antibody at 1/100,000 dilution. The proteins were visualized using Renaissance Western Blot chemiluminescence Reagent (NEN Life

Science; Boston, MA) and by exposing the membrane to autoradiography film.

Statistical Analysis

StatView 4.5 for MacIntosh (Abacus Concepts, Inc., Berkeley, CA) was used to calculate the p-values for all of the data. Briefly, the percent clot contraction (contraction assay) or the absorbance at 595nm (adhesion assay) was entered into the program. The mean, variance, standard deviation and standard error were calculated. If the variance between groups differed more than 20 fold, transformation analysis was performed on the raw data. In this paper, the function used for the contraction assays was the log (x) where x represents the % clot contraction. For the adhesion assays, the function 1/x was used to transform the data, where x represents absorbance at 595nm. Using either the transformed data or raw data, an F-test was performed as well as an ANOVA. If the ANOVA conveyed a p-value less than 0.05, than a Fisher's PLSD was calculated. A p-value less than 0.05 indicates statistical significance.

Results

Integrin Surface Expression Pattern on Human Smooth Muscle Cell Lines

The data from flow cytometry analysis indicated that both HNB18E6E7 and ductus smooth muscle cell lines expressed a number of different integrins on their surfaces, and except for $\alpha 3$, the amount of each integrin was comparable between the lines. Notably both cell lines expressed a large amount of $\beta 1$ on their surface and a small amount of $\alpha v\beta 3$ and $\alpha v\beta 5$ (Figure 2.1). In increasing amounts, HNB18E6E7 smooth muscle cells expressed the integrin $\alpha 1$, $\alpha 4$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 3$, $\alpha 5$, αv , $\alpha 2$ and $\beta 1$, while ductus smooth muscle cells expressed $\alpha 1$, $\alpha 4$, $\alpha v\beta 3$, $\alpha v\beta 5$, αv , $\alpha 5$, $\alpha 2$, $\alpha 3$ and $\beta 1$ on their cell surface. We could not detect expression of the subunits $\alpha 6$ and $\alpha 9$. By immunoprecipitating biotin labeled surface proteins, we could detect $\alpha v\beta 3$, $\alpha 5\beta 1$, αv and $\beta 1$ expression on HNB18E6E7. We could

not detect $\alpha 8$ expression (data not shown). In Table 2.1, a list of the antibodies and their antigens is presented.

Contraction of Plasma Clots by Human Smooth Muscle Cell Lines

When either the HNB18E6E7 or ductus smooth muscle cell line were incorporated into a plasma clot, they were able to contract the plasma clot to a smaller area. To determine which integrins mediate plasma clot contraction by human smooth muscle cell lines, we measured the ability of peptides that are known to bind to various integrins to inhibit clot contraction. As seen in Figure 2.2A, the linear peptides GRGDSP and GRGESP did not inhibit clot contraction by HNB18E6E7 cells. There were no significant differences among the groups (p-values > 0.05). These peptides were functional since they blocked smooth muscle cell adhesion to vitronectin at the same concentrations (data not shown). In contrast to the linear peptides, a cyclic peptide, penRGD, blocked plasma clot contraction by HNB18E6E7 approximately 75% at the highest concentration tested (p-value < 0.001) (Figure 2.2A). This peptide has been shown to block cell adhesion to vitronectin but not to fibronectin.⁹⁴ In our hands penRGD at concentrations between 250-500 μ M slightly blocked cell adhesion to fibronectin, but at 100 μ M significantly blocked adhesion to vitronectin (data not shown). The simple interpretation would be that penRGD blocks all vitronectin integrins ($\alpha v\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$), but because $\alpha v\beta 3$ is also a receptor for fibronectin, it had an effect on adhesion to fibronectin at high concentrations⁹⁴. Therefore the αv integrins⁹⁴ may be important during plasma clot contraction.

Antibodies against specific integrins were tested for their ability to inhibit plasma clot contraction. The first antibody, c7E3 Fab, was made against platelets and recognizes $\alpha IIb\beta 3$ and $\alpha v\beta 3$ ¹¹⁴. As expected this antibody blocked adhesion of the ductus smooth muscle cell line to vitronectin (data not shown). In the plasma clot contraction assay, c7E3 Fab at 150 μ g/ml blocked HNB18E6E7 mediated contraction by 50%; however, a Fisher PLSD test gave a p-value greater than 0.05 indicating that this inhibition was not statistically significant (Figure 2.2B). Likewise, a second antibody, LM609, which specifically blocks $\alpha v\beta 3$ ⁸⁵, did not significantly inhibit contraction of a plasma clot at the concentrations tested (Figure 2.2B)

nor did an αv specific antibody, L230, at 100 μ g/ml (data not shown). In contrast, a purified $\beta 1$ integrin antibody (P4C10), a purified $\alpha 5$ antibody (Mab 16) and an ascites antibody against $\alpha 5\beta 1$ (JBS5) blocked contraction of plasma clots (p -value <0.01)(Figure 2.2C). The purified mIgG antibody and the non-blocking $\beta 1$ ascites (LM534) had no effect (Figure 2.2C).

Similarly, plasma clot contraction by ductus smooth muscle cells was not inhibited by antibodies against $\alpha v\beta 3$ (data not shown). Surprisingly, the cyclic peptide, penRGD, did not inhibit contraction of plasma clots by ductus smooth muscle cells (data not shown). However, the neutralizing $\beta 1$ ascites (P4C10) did inhibit clot contraction. At 1/100 dilution, clot contraction was inhibited 100%, but a 1/100 dilution of a non-blocking $\beta 1$ ascites (LM534) had no effect (p -value <0.001) (data not shown).

Contraction of Recombinant Fibrin Clots by Human Smooth Muscle Cell Lines

To determine whether plasma molecules other than fibrin contribute to smooth muscle cell clot contraction, we incorporated recombinant fibrinogen in the established clot contraction assay. Both smooth muscle cell lines contracted the recombinant fibrin clots in the absence of exogenous plasma proteins. Contraction of recombinant fibrin clots by HNB18E6E7 was not inhibited by either penRGD (Figure 2.3A) or by L230, an anti- αv antibody (Figure 2.3A). The anti- $\alpha v\beta 3$ antibody, LM609, also did not inhibit contraction (data not shown). In contrast, contraction was inhibited by neutralizing antibodies to $\beta 1$ (P4C10) and $\alpha 5$ (Mab 16) (p -value <0.05) while a mIgG control did not block contraction (Figure 2.3B). An antibody against $\alpha 5\beta 1$ (JBS5) also inhibited recombinant fibrin clot contraction by HNB18E6E7 (data not shown). Similar results were found with ductus smooth muscle cells. LM609, an anti- $\alpha v\beta 3$ antibody, did not block recombinant fibrin clot contraction by ductus smooth muscle cells, but the anti- $\beta 1$ ascites (P4C10) at 1/100 dilution did block recombinant fibrin clot contraction (data not shown). The anti- $\alpha 5$ and anti- $\alpha 5\beta 1$ antibodies also blocked contraction of recombinant clots by ductus smooth muscle cells (data not shown).

Adhesion Assays Using Plasma Clots as a Substrate

To determine a possible mechanism of inhibition of contraction, we looked at the effect of the antibodies and peptides on adhesion of smooth muscle cells to plasma clots. Adhesion of HNB18E6E7 to plasma clots was inhibited by c7E3 Fab and LM609, anti- $\alpha v \beta 3$ antibodies (p-value<0.05), and to a greater extent by penRGD and L230, an anti- αv antibody (p-value<0.01)(Figure 2.4A). Adhesion of HNB18E6E7 to vitronectin was also inhibited significantly by c7E3, LM609, L230 and penRGD at the same concentrations (data not shown). The $\beta 1$ antibody (P4C10), $\alpha 5$ antibody (Mab 16) and ascites $\alpha 5 \beta 1$ antibody (JBS5) also inhibited adhesion of human smooth muscle cell lines to plasma clots (p-value<0.05)(Figures 2.4B, C). P4C10 at 25 $\mu\text{g/ml}$ also significantly inhibited adhesion to laminin (data not shown). The $\alpha 5 \beta 1$ and $\alpha 5$ antibodies inhibited adhesion to fibronectin, but did not inhibit cell adhesion to collagen (data not shown). Human smooth muscle cells did not adhere to either thrombin or BSA coated wells (data not shown). Comparable results were obtained with ductus smooth muscle cells using P4C10 ascites (data not shown).

Adhesion Assays Using Recombinant Fibrin Clots as a Substrate

Next we examined the ability of antibodies and cyclic peptides to inhibit adhesion of human smooth muscle cell lines to recombinant fibrin. To our surprise, adhesion to recombinant fibrin was poorly inhibited by antagonists of $\alpha v \beta 3$ (c7E3 Fab and LM609) and αv (L230) (Figure 2.5A). However, the cyclic peptide, penRGD, inhibited adhesion 50% (p-value<0.05) (Figure 2.5A). Furthermore, the $\beta 1$ neutralizing antibody, the $\alpha 5$ neutralizing antibody and $\alpha 5 \beta 1$ antibody all had a statistically significant inhibitory effect (p-value<0.05)(Figures 2.5B, C). Similar results were found using the ductus smooth muscle cell line. Cell adhesion to recombinant fibrin was inhibited by antibodies against $\alpha 5$, $\alpha 5 \beta 1$ and $\beta 1$, but not by antibodies against $\alpha v \beta 3$ (data not shown).

Adhesion Assay Using an Antibody Against Human Vitronectin

The differential effect of anti- $\alpha v \beta 3$ antibodies on smooth muscle cells adherence to plasma clots versus recombinant fibrin clots was puzzling. To determine whether the ligand for $\alpha v \beta 3$ in plasma clots was vitronectin, we decided to treat the matrix with an antibody against human vitronectin, one

of the main ligands for $\alpha v \beta 3$. The matrix was preincubated with either the anti human vitronectin polyclonal antibody or a rabbit IgG control. We found that adhesion of HNB18E6E7 was significantly inhibited by this antibody at 184 μ g/ml and 337 μ g/ml while the IgG control had no effect at the same concentrations (p-value <0.01) (Figure 2.6). This suggests that in plasma clots, vitronectin is one of the substrates that the HNB18E6E7 smooth muscle cells are recognizing. This antibody also inhibited HNB18E6E7 adhesion to vitronectin, but not to whole plasma (data not shown).

Fibrin Clot Contraction Assay in the Presence of Cycloheximide

To determine if protein synthesis is required for fibrin clot contraction, HNB18E6E7 smooth muscle cells were treated with 80 μ g/ml of cycloheximide for one hour. In both plasma and recombinant clot contraction assays, the ability of the cells to contract the clot was not effected by the presence of cycloheximide (Figure 2.7). To characterize whether cells were secreting fibronectin during the assay, the supernatant or clot was removed from the test tube at the conclusion of the experiment and electrophoresed on a SDS polyacrylamide gel. Following transfer to a PVDF membrane the presence of human fibronectin was detected using an anti-human fibronectin antibody which does not cross react with bovine fibronectin. Fibronectin was detected in the samples that were treated with cycloheximide, indicating that secretion occurred (Figure 2.8). At the same time, a metabolic labeling experiment was carried out in the presence and absence of cycloheximide. The results showed that protein synthesis was decreased by 95% during the time frame of the contraction assay (Figure 2.9A). To test whether cell-fibrin interaction via $\alpha 5 \beta 1$ was dependent on protein synthesis, a plasma clot contraction assay was carried out in the presence of cycloheximide and anti- $\alpha 5$ antibody. Anti- $\alpha 5$ antibody still inhibited plasma clot contraction in the presence of cycloheximide (Figure 2.9B). Therefore, $\alpha 5$ may be interacting with a protein present in the initial plasma clot or with a secreted matrix protein.

34
Discussion

While it is logical to assume that narrowing of atherosclerotic or restenotic vessels is a simple result of accumulation of plaque material, several studies in human and animals have failed to show a correlation between intimal plaque mass and stenosis.^{2, 3, 73, 74} We have been intrigued by the possibility that intra-mural fibrin, a prominent component of advanced plaques^{19, 47} and a critical component of the provisional matrix required for wound healing,⁷⁶ might play a role in wound contraction and thus in narrowing of injured vessels. This hypothesis is supported by studies by Courtman *et al.*³⁷ who used a factor VIIa inhibitor in a balloon injury rabbit model. The VIIa inhibitor prevented the generation of thrombin, and thus less fibrin was deposited on and in the vessel of the treated animals. The control animals but not the treated animals showed vascular narrowing. Importantly, the areas of the media and intima in the two groups did not differ, suggesting that the difference in lumen size was not due to change in mass but to remodeling. These data suggest that fibrin could play a role in vascular narrowing.

The data presented in this paper demonstrate that human smooth muscle cell lines are able to contract clots composed of either plasma that has been depleted of fibronectin, or recombinant fibrinogen. Because $\alpha v \beta 3$ has been reported to be a fibrin receptor on nucleated cells,^{57, 115} we expected that LM609 and 7E3 Fab, two antibodies against $\alpha v \beta 3$, would inhibit smooth muscle cell contraction of fibrin clots. Remarkably, antibodies against $\alpha v \beta 3$ failed to significantly inhibit contraction of fibrin clots. We did not see any inhibition even at 100 $\mu\text{g}/\text{ml}$ of LM609. However, the cyclic peptide (penRGD), the $\beta 1$ antibody (P4C10), the $\alpha 5$ antibody (Mab 16) and the $\alpha 5 \beta 1$ antibody (JBS5) inhibited contraction of plasma clots by human smooth muscle cell lines. With recombinant fibrin clots, P4C10, Mab 16 and JBS5 inhibited smooth muscle cell mediated contraction. These data suggest that contraction of fibrin clots by human smooth muscle cell lines is mainly mediated by the $\alpha 5 \beta 1$ integrin.

In concurrence with the contraction assay data, antibodies P4C10, Mab 16, and JBS5 also inhibited cell adhesion to both plasma clots and recombinant fibrin clots. Furthermore, cell adhesion to plasma clots was

inhibited by the anti- $\alpha v \beta 3$ antibodies (LM609 and 7E3 Fab), an anti- αv antibody (L230), the cyclic peptide (penRGD) and an anti-vitronectin antibody suggesting that vitronectin may be the ligand for $\alpha v \beta 3$ in this system. In contrast, adhesion to recombinant clots was not inhibited by anti- $\alpha v \beta 3$ antibodies. This result is not surprising since vitronectin should not be present in recombinant fibrin. However, the cyclic peptide, penRGD, did prevent cell adhesion to recombinant fibrin at $500 \mu\text{M}$. We found that at high concentrations, penRGD will inhibit smooth muscle cell adhesion to fibronectin. Overall, the data suggest that adhesion to recombinant fibrin is $\alpha 5 \beta 1$ dependent while adhesion to plasma clots involves $\alpha v \beta 3$ and $\alpha 5 \beta 1$. Thus adhesion to recombinant fibrin clots may be mediated by a subset of the integrins involved in plasma clot contraction and adhesion.

The differential effect of anti- $\alpha v \beta 3$ antibodies on plasma clot contraction versus cell adhesion may be due to the inherent differences in the two processes. Human smooth muscle cells will spread when placed within a plasma clot, but remain rounded within a recombinant fibrin clot (unpublished data, 1997). In both situations, the cells will contract the fibrin clot. In contrast, during adhesion to both plasma clots and recombinant fibrin clots, the cells achieve a spread morphology. Furthermore we have observed that anti- $\alpha v \beta 3$ antibodies prevent cell spreading in both assay systems. This may not affect the cell's ability to contract a fibrin clot, but it may affect the cell's ability to adhere to a clot.

In our assays, we have also seen conflicting results between the anti- αv antagonists. The peptide (penRGD) did not block contraction, but it did block adhesion to recombinant clots. In contrast, the anti- αv antibody (L230) did not inhibit either smooth cell adhesion to or contraction of recombinant fibrin clots. The two different results using anti- αv antagonists may be due to the different epitopes that are recognized by the peptide and the antibody. We also saw that penRGD blocked plasma clot contraction while LM609 did not have an effect. This could be due to the fact that penRGD blocks all αv integrins while LM609 only blocks $\alpha v \beta 3$. If vitronectin is playing a role in cell interaction with plasma clots, then LM609 may not have a significant effect due to the availability of the other two αv integrins to interact with the matrix.

The idea that a $\beta 1$ integrin is a fibrin receptor is not novel. Three groups have reported that cells will adhere to fibrinogen via a $\beta 1$ integrin.⁸¹⁻⁸³ Marshall *et al.* demonstrated that adhesion of $\alpha v\beta 3$ negative human melanoma cells to fibronectin depleted fibrinogen was inhibited by antibodies against αv and $\beta 1$, thus implicating $\alpha v\beta 1$ as the integrin mediating this interaction. Human melanoma cells that express high levels of both $\alpha v\beta 1$ and $\alpha v\beta 3$ did not adhere to fibronectin depleted fibrinogen in the presence antibodies against $\alpha v\beta 3$. Anti- $\beta 1$ antibodies had no effect.⁸¹ Neugebauer *et al.* showed that adhesion of a chicken myeloblast cell line, which express $\alpha v\beta 3$ and not $\alpha v\beta 1$, to commercial fibrinogen was inhibited by an anti- $\alpha v\beta 3$ antibody and a $\beta 1$ antibody.⁸² Suehiro *et al.* reported that endothelial cells bind directly to fibrinogen via $\alpha 5\beta 1$ in the presence of manganese. However, in the presence of calcium, integrin mediating endothelial interaction with fibrinogen was through $\alpha v\beta 3$.⁸³

Several factors distinguishes these three studies from ours. First, fibrinogen and not fibrin was the matrix of choice and it was not determined whether the cells were secreting any matrix proteins. Second, these investigations utilized either commercial fibrinogen, which is likely to be contaminated with plasma proteins, or gelatin sepharose treated plasma. Gelatin sepharose treated plasma has been used to effectively isolate cell-fibrinogen interaction because cells can adhere to fibrinogen via fibronectin. Therefore the source of fibrinogen could also influence any results. In our studies, we have used recombinant fibrinogen, which is initially free of contaminating plasma proteins, and may be a better source of "pure" fibrinogen. We have also repeated the contraction assays using whole plasma, a more physiologically relevant source of fibrinogen, and found inhibition by both Mab 16 and P4C10 (unpublished data, 1997). Therefore, we conclude that $\alpha 5\beta 1$ may participate in smooth muscle cell adhesion and contraction of physiologically relevant plasma clots.

Whether the smooth muscle cells are utilizing $\alpha 5\beta 1$ to directly interact with fibrin is still unclear. We found that the addition of cycloheximide to the cell culture did not affect the cells' ability to contract either a plasma clot or a recombinant fibrin clot; however, we also found that even in the presence of cycloheximide, the cells secreted fibronectin.

What role fibronectin plays in smooth muscle cell contraction and adhesion to fibrin clots is still being investigated. The linear GRGDSP peptide and the cyclic penRGD peptide did not inhibit recombinant fibrin clot contraction. This could indicate that $\alpha 5\beta 1$ on smooth muscle cells is not binding to an RGD site on fibronectin during recombinant fibrin clot contraction. Thus we need to consider the possible sites of fibrin that may be binding sites for integrins. It should be noted that Suehiro *et al.* determined that endothelial cells bind the C terminal RGD site on the alpha chain of fibrinogen,⁸³ while Smith *et al.* demonstrated that this RGD site is not required to support endothelial cell-mediated clot retraction.¹¹⁶

Finally, the accuracy of the representation of integrins in cultured smooth muscle cells as a model for integrins in smooth muscle cells *in vivo* is an important issue. Surprising little is known about the profile of integrins expressed by vascular smooth muscle cells *in vivo*.¹¹⁷⁻¹²¹ Hoshiga *et al.* found that smooth muscle cells of both atherosclerotic and non atherosclerotic coronary arteries expressed diffuse but extensive amounts of $\alpha v\beta 3$ in the media.⁶⁵ This study also showed that $\alpha v\beta 3$ is expressed in some smooth muscle cells that have accumulated in the intima but such cells are relatively rare. Recently, Dr. Yuji Ikari has seen $\alpha 5\beta 1$ expression on smooth muscle cells in atherosclerotic lesions⁶⁶. In contrast to the *in vivo* studies, cultured smooth muscle cells express variable levels of $\alpha v\beta 3$.⁶⁴ This difference in integrin expression may be due to culture conditions as it is known that the extracellular matrix affects the integrin expression pattern on cultured cells.¹²² The two cell lines examined here were chosen because they consistently express $\alpha v\beta 3$ as well as a selection of $\beta 1$ integrins.

Our data is not the first to study the interactions of smooth muscle cells with fibrin. Barbieri *et al.* showed that bovine smooth muscle cells contract fibrin clots, and Naito *et al.* have investigated bovine smooth muscle cell attachment and migration on purified bovine fibrin obtained commercially.^{84, 123, 124} We have shown that human smooth muscle cell lines are able to contract a fibrin clot and that certain integrins mediate this interaction. Surprisingly, antibodies to the $\alpha v\beta 3$ integrin, a known receptor for fibrin, did not significantly inhibit contraction, while antibodies to $\alpha 5, \beta 1$

and $\alpha 5\beta 1$ significantly blocked contraction. This advocates that $\alpha 5\beta 1$ integrin is important in this process. The fact that the smooth muscle cells are able to contract a fibrin matrix *in vitro* suggest that this event can occur *in vivo* and thus contribute to vessel narrowing during atherosclerosis progression.

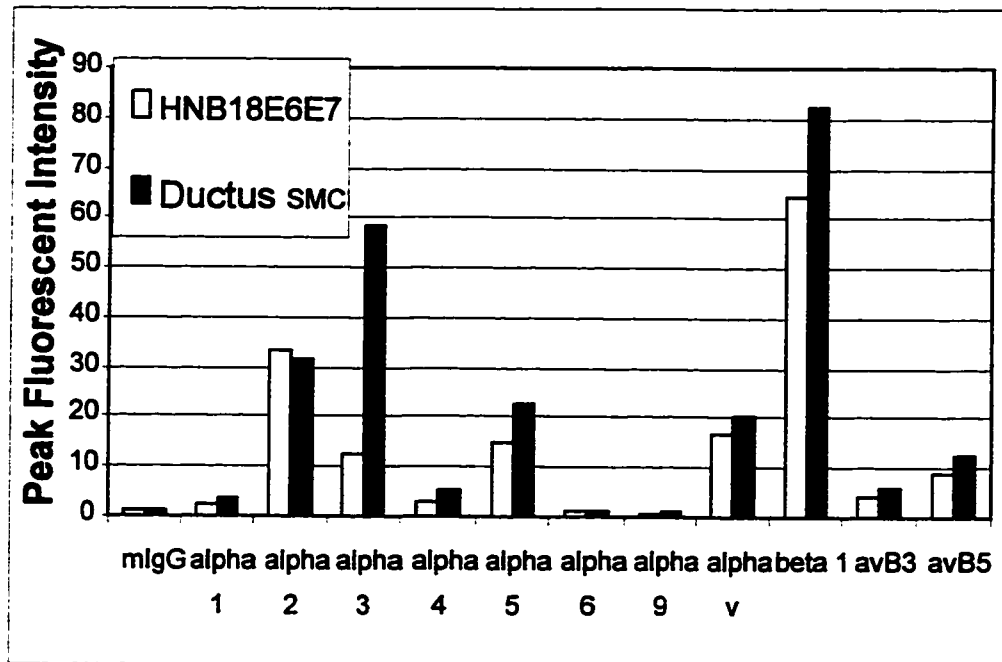
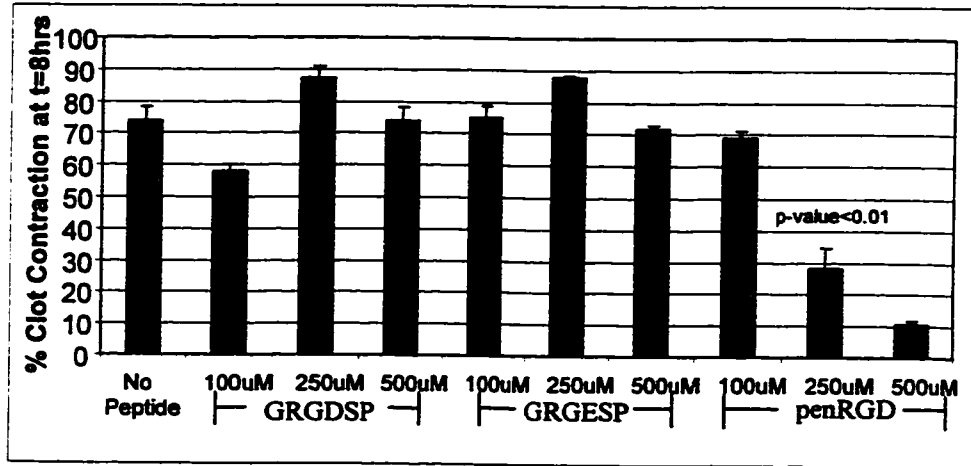
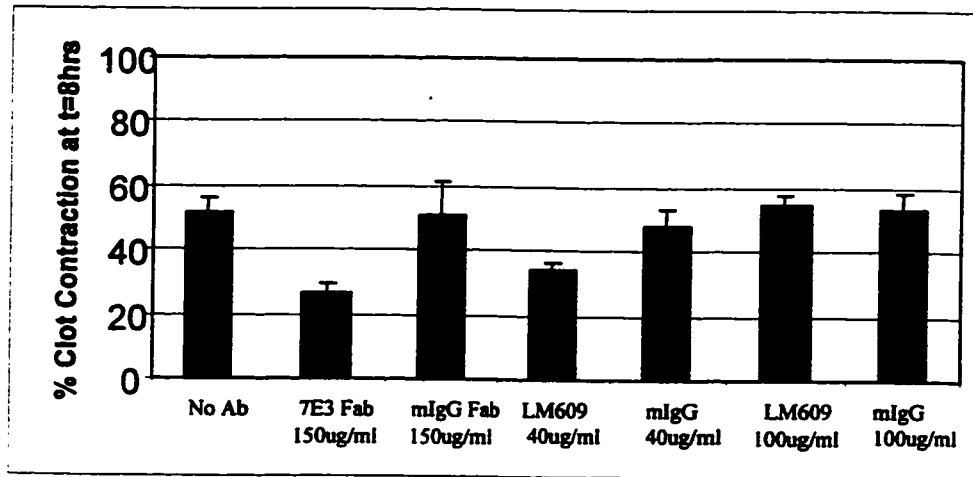
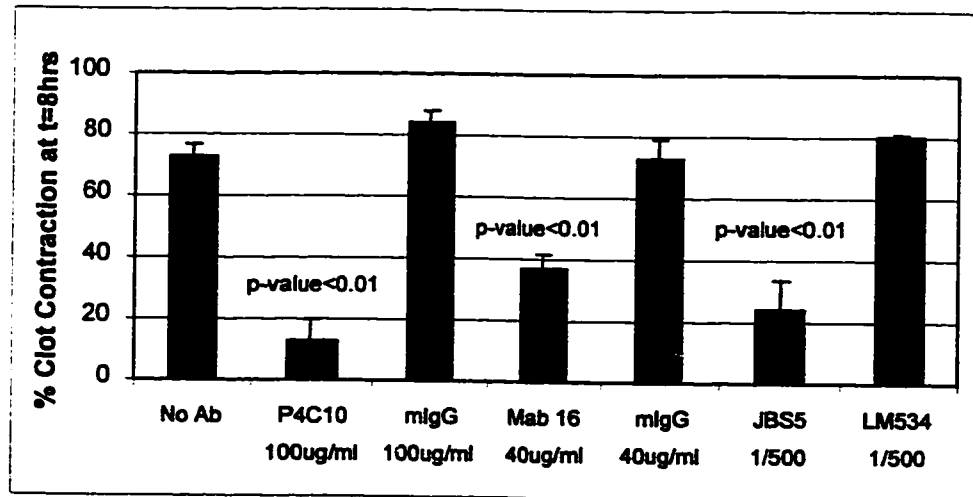


Figure 2.1: Integrin surface expression pattern on HNB18E6E7 and Ductus smooth muscle cells.

(A) Cells were incubated with the following primary antibodies: mIgG, 5E8D9 (anti-alpha 1), P1E6 (anti-alpha 2), P1B5 (anti-alpha 3), P4G9 (anti-alpha 4), P1D6 (anti-alpha 5), J1B5 (anti-alpha 6), Y9A2 (anti-alpha 9), 1980 (anti-alpha v), P4C10 (anti-beta 1), LM609 (anti- $\alpha v\beta 3$), and P1F6 (anti- $\alpha v\beta 5$), followed by a phycoerythrin conjugated anti-mouse IgG. Peak fluorescent intensity was measured by flow cytometry.

Figure 2.2: Effect of peptide antagonists and anti-integrin antibodies on plasma clot contraction by HNB18E6E7.

Human smooth muscle cells were untreated or treated with either (A) 100 μM , 250 μM or 500 μM GRGDSP/GRGESP peptides or 100 μM , 250 μM or 500 μM penRGD peptides (B) 150 $\mu\text{g/ml}$ of 7E3 FAB or mIgG FAB, 40 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$ of LM609 (anti- $\alpha\text{v}\beta\text{3}$) or mIgG (C) 100 $\mu\text{g/ml}$ of P4C10 (anti- β1) or mIgG, 40 $\mu\text{g/ml}$ of Mab 16 (anti- α5) or mIgG, or 1/500 dilution of JBS5 (anti- $\alpha\text{5}\beta\text{1}$) and 1/500 dilution of LM534 (non-blocking anti- β1) for 30 minutes at room temperature. Fibronectin depleted human plasma and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 8 hours. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3).

A**B****C**

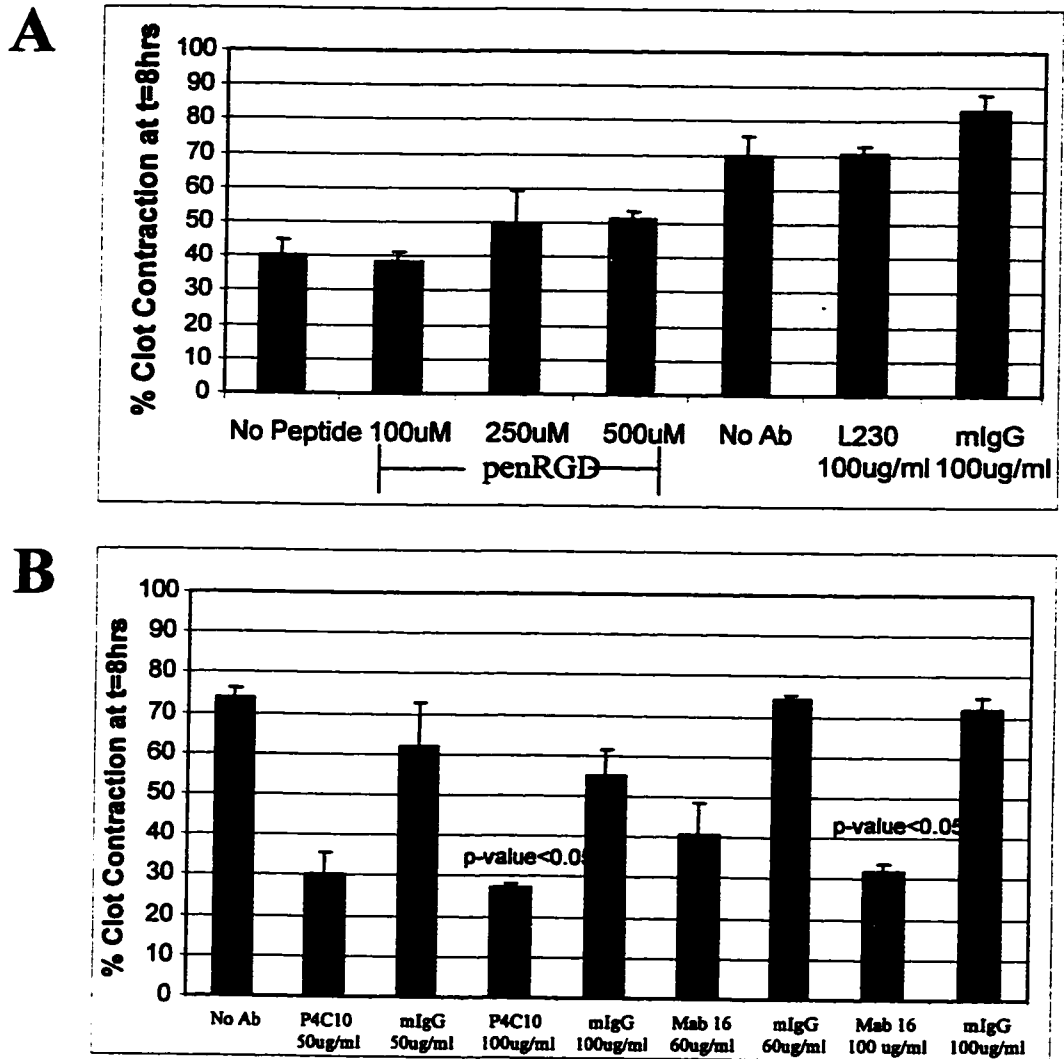


Figure 23: Effect of a cyclic-peptide antagonist and anti-integrin antibodies on recombinant clot contraction by HNB18E6E7.

Human smooth muscle cells were untreated or treated with either (A) 100 μ M, 250 μ M or 500 μ M of penRGD or 100 μ g/ml of L230 (anti- α v) or mIgG (B) 50 μ g/ml and 100 μ g/ml of P4C10 (anti- β 1) or mIgG, 60 μ g/ml or 100 μ g/ml of Mab 16 (anti- α 5) or mIgG for 30 minutes at room temperature. Recombinant fibrinogen and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 8 hrs. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3).

Figure 2.4: Inhibition of cell adhesion to plasma using anti-integrin antibodies

(A) Inhibiting adhesion of HNB18E6E7 smooth muscle cells to plasma clots using anti- $\alpha v \beta 3$ antibodies (LM609 and c7E3 Fab), anti- αv antibody (L230) and a cyclic peptide. Plasma and thrombin were coated onto a 96 well plate overnight for 24 hrs at 4°C. Human smooth cells were preincubated for 15 minutes at room temperature with either 10 $\mu\text{g/ml}$ of LM609, 100 $\mu\text{g/ml}$ of c7E3 Fab or mIgG Fab, 40 $\mu\text{g/ml}$ of L230 or mIgG or 500 μM of penRGD. The cells were allowed to adhere to the substrate for 1 hour at 37°C and non-adhering cells were washed off with PBS. The adhering cells were fixed with 4% paraformaldehyde and stained with toluidine blue. The color intensity was measured by an ELISA reader at 595nm. Values represent the mean divided by the mean of the No Ab control \pm standard error (n=3). **(B)**

Inhibiting adhesion of HNB18E6E7 to plasma clots using the anti- $\beta 1$ antibody, P4C10. Procedure is the same as in figure 4A except that human smooth muscle cells were preincubated for 15 minutes at room temperature with 20 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$ and 60 $\mu\text{g/ml}$ of P4C10 and mIgG. Values represent the mean divided by the mean of the No Ab control \pm standard error (n=3).

(C) Inhibiting adhesion of HNB18E6E7 to plasma clots using the anti- $\alpha 5$ antibody, Mab 16, and the anti- $\alpha 5 \beta 1$ antibody, JBS5. Procedure is the same as in figure 4A except that human smooth muscle cells were preincubated for 15 minutes at room temperature with either 20 or 40 $\mu\text{g/ml}$ of Mab 16 or mIgG, or 1/200 or 1/500 dilution of JBS5 or LM534. Values represent the mean divided by the mean of the No Ab control \pm standard error (n=3).

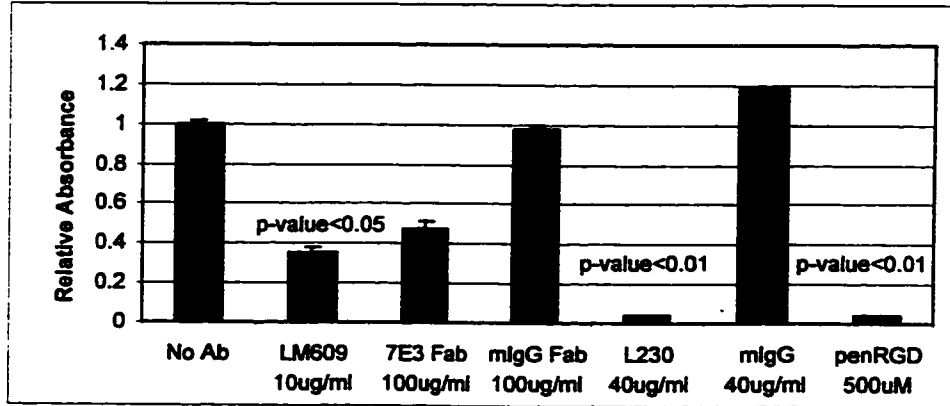
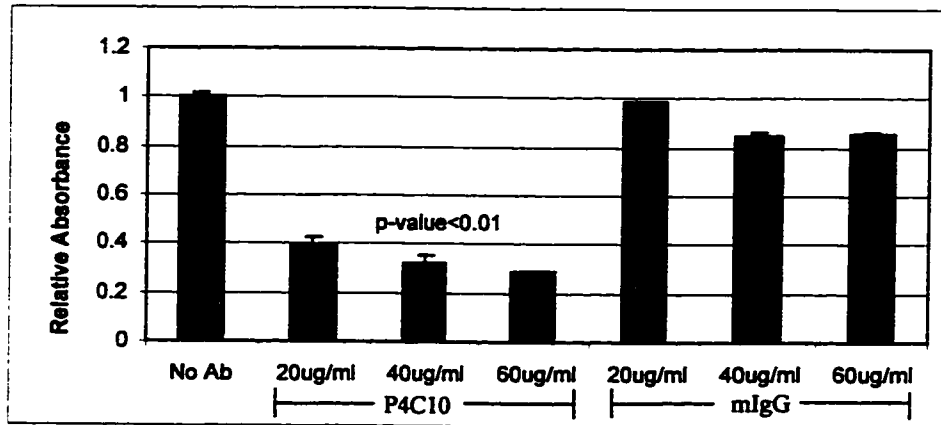
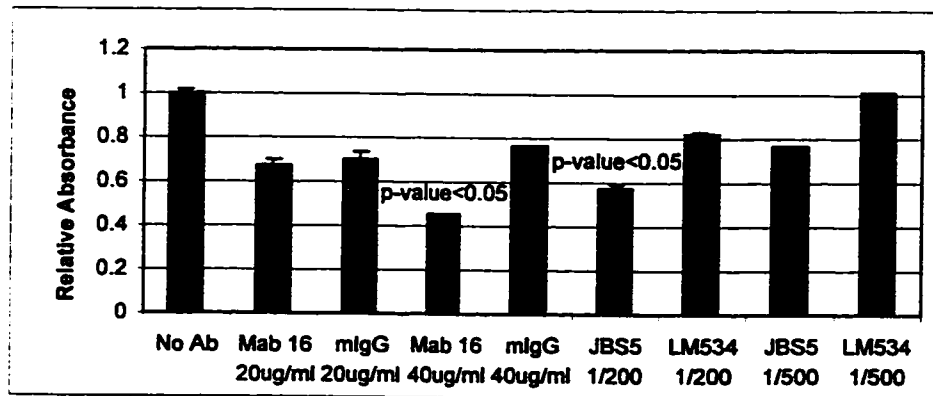
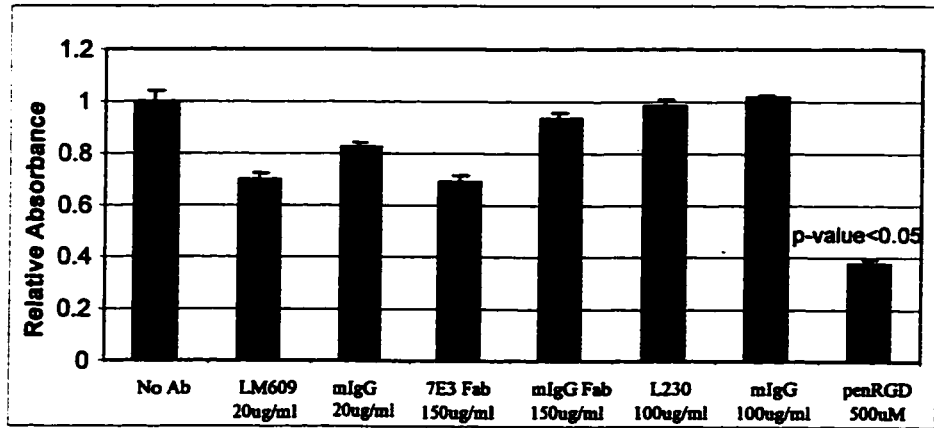
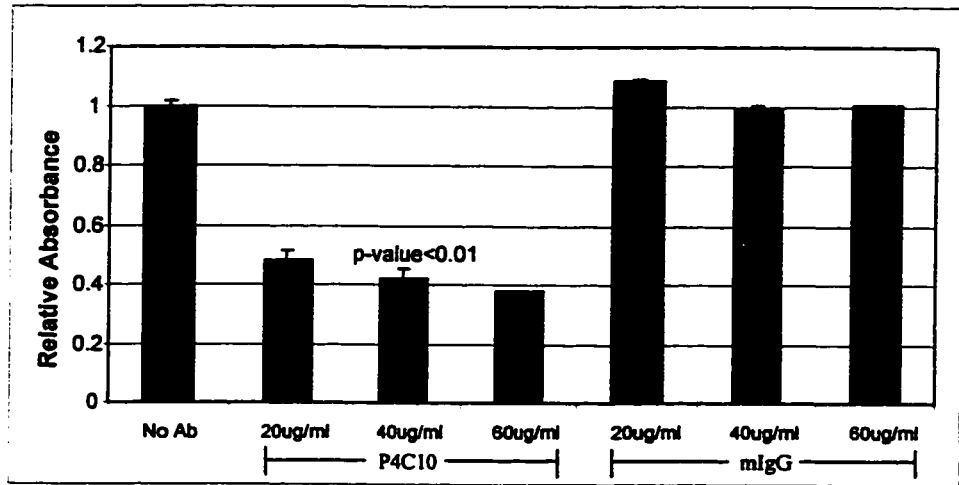
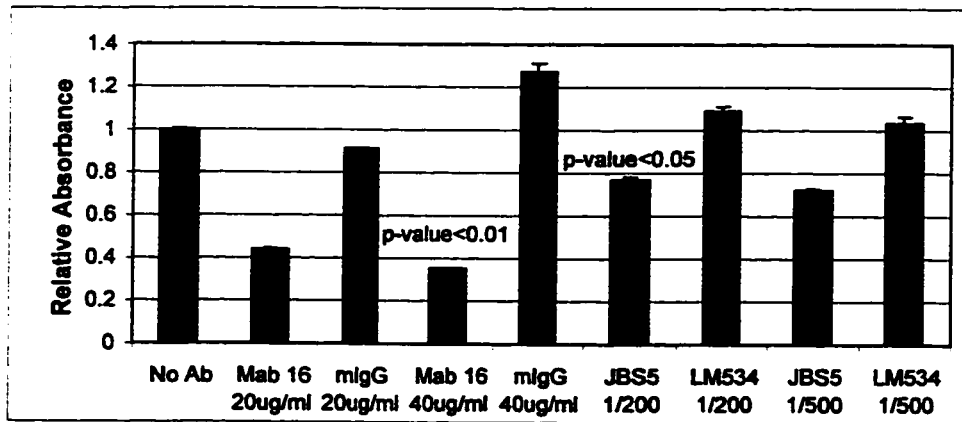
A**B****C**

Figure 2.5: Inhibition of cell adhesion to recombinant fibrin using anti-integrin antibodies

(A) Inhibiting adhesion of HNB18E6E7 smooth muscle cells to recombinant fibrin using anti- α v β 3 antibodies (LM609 and c7E3 Fab), an anti- α v antibody (L230) or a cyclic peptide (penRGD). Recombinant fibrin and thrombin were coated onto a 96 well plate overnight for 24 hrs at 4°C. Human smooth cells were preincubated for 15 minutes at room temperature with either 20 μ g/ml of LM609 or mIgG, 150 μ g/ml of c7E3 Fab or mIgG or 500 μ M of penRGD peptide. The cells were allowed to adhere to the substrate for 1 hour at 37°C, and non-adhering cells were washed off with PBS. The adhering cells were fixed with 4% paraformaldehyde and stained with toluidine blue. The color intensity was measured by an ELISA reader at 595nm. Values represent the mean divided by the mean of the No Ab control \pm standard error (n=3). **(B)** Inhibiting adhesion of HNB18E6E7 smooth muscle cells to recombinant fibrin using the anti- β 1 antibody, P4C10. The procedure is the same as in 5A except that human smooth cells were preincubated for 15 minutes at room temperature with 20 μ g/ml, 40 μ g/ml and 60 μ g/ml of P4C10 and mIgG. Values represent the mean divided by the mean of the No Ab control \pm standard error (n=3). **(C)** Inhibiting adhesion of HNB18E6E7 smooth muscle cells to recombinant fibrin using the anti- α 5 antibody, Mab 16, and the anti- α 5 β 1 antibody, JBS5. The procedure is the same as in 5A except human smooth cells were preincubated for 15 minutes at room temperature with 20 or 40 μ g/ml of Mab 16 or mIgG, or 1/200 or 1/500 dilution of JBS5 or LM534. Values represent the mean divided by the mean of the No Ab control \pm standard error (n=3).

A**B****C**

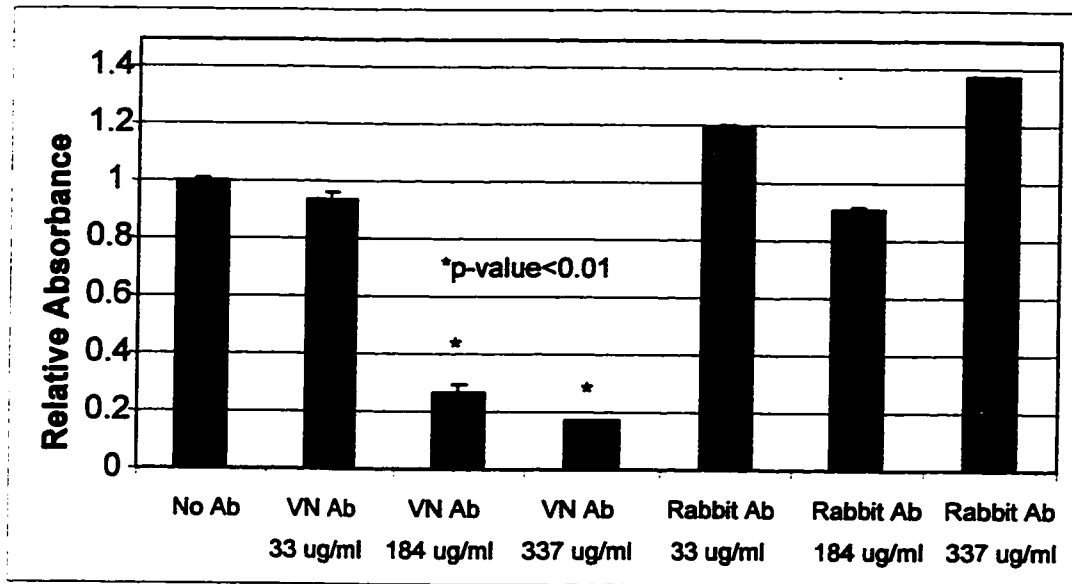


Figure 26: Effect of an anti-human vitronectin polyclonal antibody on HNB18E6E7 adhesion to plasma clots.

The procedure is similar to the one in figure 5 except that plasma clots were preincubated for 15 minutes at room temperature with 33 $\mu\text{g}/\text{ml}$, 184 $\mu\text{g}/\text{ml}$ or 337 $\mu\text{g}/\text{ml}$ of vitronectin polyclonal antibody or rabbit IgG. Untreated cells were then added to each well. Values represent the mean divided by the mean of the No Ab control \pm standard error (n=3).

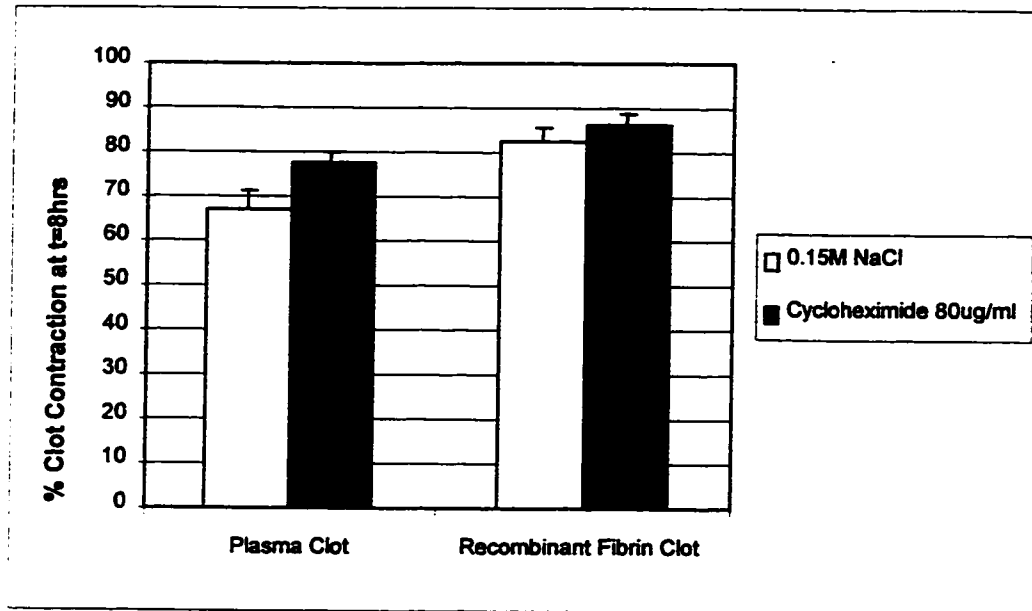


Figure 2.7: Effect of cycloheximide on plasma clot and recombinant fibrin clot contraction by HNB18E6E7.

Cells were preincubated with 80 $\mu\text{g}/\text{ml}$ of cycloheximide for 1 hour. Either fibronectin depleted human plasma or recombinant fibrinogen and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 8 hrs. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3).

- | | |
|-------------------------------------|----------------------------------|
| 1. Human Fibronectin | 7. Supernatant (+Cycloheximide) |
| 2. Supernatant (+ cycloheximide) | 8. Supernatant |
| 3. Supernatant | 9. Cell Lysate (+ Cycloheximide) |
| 4. Cell lysate (+ cycloheximide) | 10. Cell Lysate |
| 5. Cell lysate (Recombinant Fibrin) | 11. FN depleted Plasma Only |
| 6. Recombinant Fibrin Only | |

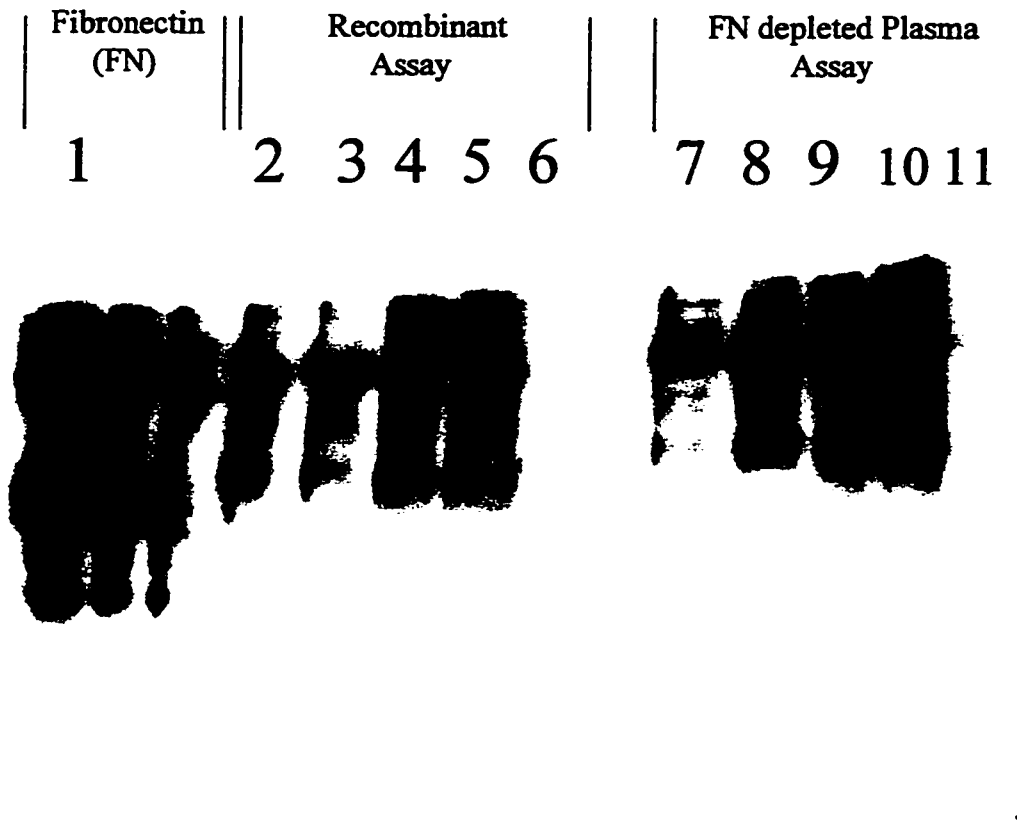


Figure 2.8: Presence of fibronectin in the supernatant and the cellular clot in the presence and absence of cycloheximide.

Either the supernatant or the clot was removed from the experiment in figure 6. The samples were electrophoresed on a 5% SDS-PAGE, transferred to a membrane and probed with an anti-human fibronectin antibody and a goat-anti-mouse antibody.

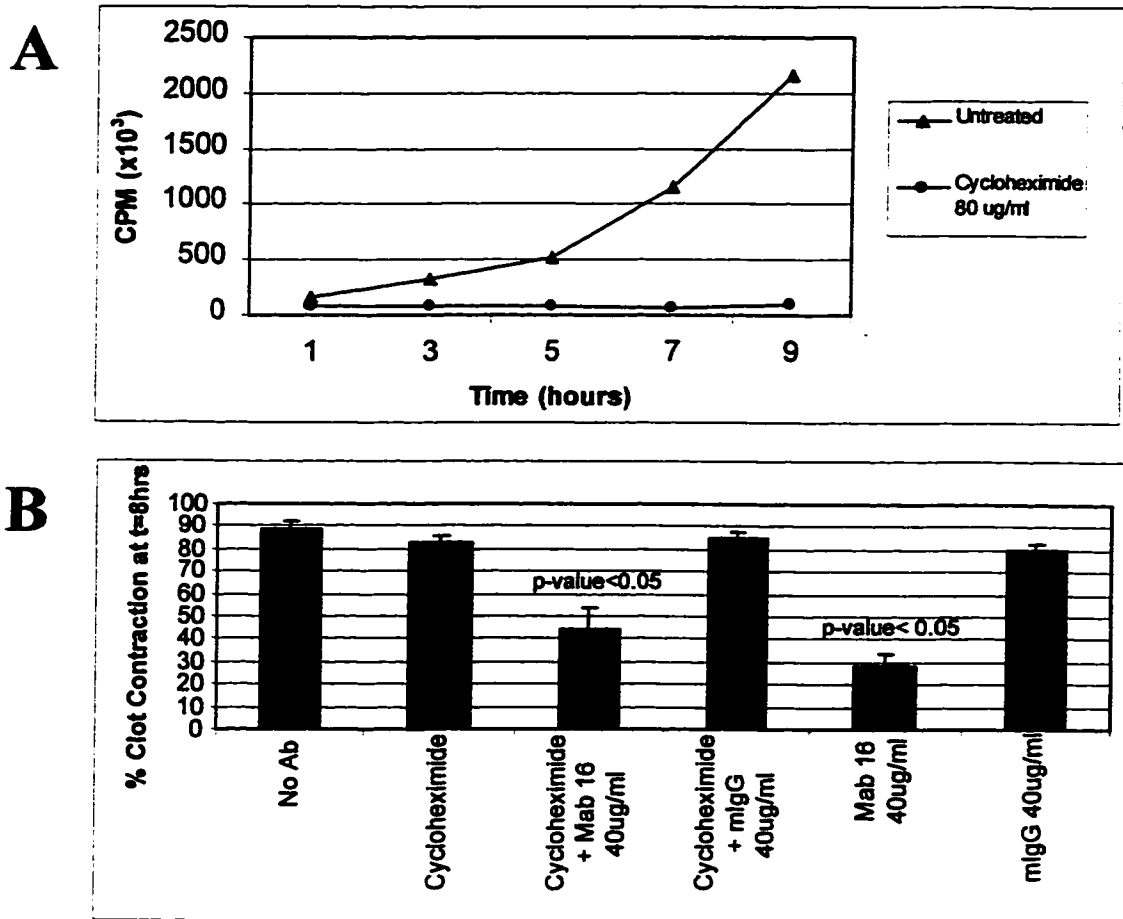


Figure 2.9: Effect of cycloheximide on protein synthesis and clot contraction

(A) Effect of cycloheximide on protein synthesis. Smooth muscle cells were incubated with 80 $\mu\text{g}/\text{ml}$ of cycloheximide and ^{35}S -methionine in methionine-free DME media for 9 hours. At 1, 3, 5, 7 and 9 hours, cells were lysed and the supernatant was measured in a scintillation counter for the presence of ^{35}S -methionine. (B) Effect of cycloheximide and anti- $\alpha 5$ antibody, Mab 16, on plasma clot contraction. Smooth muscle cells were incubated with 80 $\mu\text{g}/\text{ml}$ of cycloheximide for 1 hour and 40 $\mu\text{g}/\text{ml}$ of anti- $\alpha 5$ antibody, Mab 16, for 30 minutes at room temperature. Fibronectin depleted plasma and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 8 hrs. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3).

Table 2.1. List of antibodies and their antigens

| Antibody | Antigen |
|-----------------|---|
| LM609 | $\alpha v \beta 3$ |
| c7E3 Fab | $\alpha v \beta 3$ and $\alpha IIb \beta 3$ |
| L230 | αv |
| Mab 1980 | αv |
| P4C10 | $\beta 1$ |
| DE9 | $\beta 1$ |
| LM534 | $\beta 1$ |
| 5E8D9 | $\alpha 1$ |
| P1E6 | $\alpha 2$ |
| P1B5 | $\alpha 3$ |
| P4G9 | $\alpha 4$ |
| Mab 16 | $\alpha 5$ |
| JBS5 | $\alpha 5 \beta 1$ |
| JIB5 | $\alpha 6$ |
| Y9A2 | $\alpha 9$ |
| P1F6 | $\alpha v \beta 5$ |
| Polyclonal VN | human vitronectin |
| Ascites Clone I | human fibronectin |

**Chapter 3: Fibronectin Plays a Critical Role in Human
Smooth Muscle Cell Interaction with Fibrin**

**This chapter is part of a manuscript that will be submitted for publication by
Karen O Yee, Michael M. Rooney, Susan T. Lord and Stephen M. Schwartz.**

Introduction to Chapter 3

The common belief is that vascular narrowing in atherosclerosis is caused by an increase of cells, lipids and extracellular matrix molecules on the vessel wall. However, both primary and restenotic vessels have been shown to be able to accommodate an increase in lesion mass by adaptive remodeling to maintain normal blood flow by enlarging the cross sectional areas of the vessel. This process is called compensatory enlargement or adaptive remodeling^{2, 3, 74}. The failure of vessels to remodel or pathological remodeling to actively narrow the vessel will lead to lumen narrowing and clinical symptoms of atherosclerosis.

We have suggested that failure to remodel could be due to smooth muscle cell interactions with fibrin during the wound healing that occurs following plaque rupture^{15, 37}. Courtman *et al.* demonstrated the detrimental effect of fibrin on vessel lumen area during healing of injured vessels³⁷. The abdominal aorta of a rabbit was twice injured using a balloon catheter and treated with a Factor VIIa inhibitor which prevented the formation of thrombin and thus fibrin. This paper found that the treated vessels had a larger lumen diameter than the untreated vessels without a difference in the intima or media area. Thus fibrin was involved not only in the failure of vessels to remodel after injury, but in an active narrowing that might be considered pathological remodeling. Such a role for fibrin in the vascular response to injury might explain the well known role of fibrinogen levels and other pre-coagulants as risk factors in atherosclerosis. Therefore we considered the possibility that fibronectin was the ligand, perhaps acting as a bridge to fibrin (REF). Our clot contraction studies were done in gelatin sepharose treated plasma and recombinant fibrin, situations where we would not expect fibronectin to be present, other than trace amounts of hamster fibronectin used to produce the recombinant fibrinogen in the transfected CHO cells.

However, because integrin antagonists have been introduced as therapeutic reagents, we attempted to define the integrins responsible for human smooth muscle cell interaction with fibrin, an extracellular matrix found in all wounds. We found that this interaction is mediated by the $\alpha 5\beta 1$ integrin¹²⁵. This integrin is a receptor for the extracellular matrix

molecule, fibronectin, though recently one group has demonstrated that in the presence of manganese $\alpha 5\beta 1$ can be a receptor for fibrinogen on endothelial cells^{83, 121}. Fibrinogen, however, did not appear to be the receptor in our studies because we demonstrated that fibronectin is secreted by the cells during its interaction with fibrin, even in the presence of cycloheximide. This suggests that there are internal stores of fibronectin in human smooth muscle cells and these stores are released during human smooth muscle cell interaction with fibrin.

Fibronectin is a large glycoprotein that is secreted into the blood by hepatocytes¹²⁶ and is also secreted by several cells types such as endothelial cells, fibroblasts, chondrocytes and glial cells¹²⁷. Fibronectin is found in the matrix surrounding the cells as well as in the basement membrane. It is a homodimer linked by disulfide bonds at the carboxyl terminus and within each subunit exists a number of functional domains. Fibronectin has a collagen binding domain, cell binding domain that includes the well known integrin recognition motif RGD, a fibrin and transglutaminase interaction region as well as glycosaminoglycan-binding domains¹²⁷⁻¹²⁹. Thus fibronectin could play a key role in this process.

Two different post-translational types of fibronectin exist, plasma and cellular¹³⁰. They differ in their isoelectric point, solubility, number of subunits linked together by disulfide bonds and their size^{126, 128}. Cellular fibronectin is slightly larger than plasma fibronectin because of the existence of the ED-A and ED-B domains¹²⁸. Cellular fibronectin also interacts with the microfilament component of the cells¹³¹. Both plasma and cellular fibronectin will promote cell adhesion, cell spreading and cell migration^{96, 132, 133}. There are monoclonal antibodies that are able to distinguish between the two forms¹³⁰. The receptors for fibronectin includes the integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ which are expressed by numerous cells types including endothelial cells, fibroblasts, melanoma and smooth muscle cells^{121, 133, 134}.

There is a precedent in thrombosis for proposing that an interaction of fibrin and fibronectin may be important during wound healing. Platelets adhere to fibrin during the initial injury and release fibronectin from their alpha granules¹³⁵. Plasma fibronectin will also be caught up in the fibrin meshwork of the thrombus. Fibrin interacts with fibronectin non-

covalently, but can also be cross-linked to fibrin by transglutaminase or Factor XIIIa¹³⁶. The fibronectin binding site on fibrin(ogen) is on the A α chain and it is also the same site where Factor XIIIa can cross-link fibronectin to fibrin^{137, 138}. On fibronectin, there are two fibrin(ogen) binding sites on the N and C terminus of the molecule^{129, 139}. It would be logical to assume that in healing wounds, fibronectin and fibrinogen will be co-localized. Indeed cells attach and spread better on fibronectin-fibrin matrices than on fibrin alone^{140, 141}. Therefore, smooth muscle cells in a ruptured plaque could interact with fibrin through fibronectin.

In the work presented here, we extend our previous studies on human smooth muscle cell interaction with fibrin. Using both monoclonal and polyclonal antibodies we demonstrate that smooth muscle cell adhesion and contraction of whole plasma clots, gelatin sepharose treated plasma clots and recombinant fibrin clots are dependent on fibronectin. In particular we show that both cellular and plasma fibronectins play a role in smooth muscle cell interaction with fibrin and that this fibronectin could be acting as a bridge molecule between the fibrin matrix and the smooth muscle cell. Because fibronectin and fibrin will often be co-localized in atherosclerotic plaques and areas of thromboses *in vivo*, we believe that the work presented here highlights the importance of fibronectin in the overall scheme of smooth muscle interaction with fibrin in the plaque. This component in the equation should be taken into consideration when designing pharmaceutical reagents for the prevention of atherosclerosis progression.

Materials and Methods

Antibodies and Reagents

Brefeldin A was purchased from Calbiochem (La Jolla, CA). Anti-human fibronectin antibody (clone 3E1) was purchased from Gibco/BRL (Gaithersburg, MD)⁹⁵. This antibody does not recognize bovine fibronectin. Human fibronectin was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Human Newborn 18 smooth muscle strain has been described previously and was originally derived from the aorta of a newborn infant (2-day old) autopsy.^{107, 125} This strain has been transfected with a plasmid containing the type 16 human papilloma virus proteins E6 and E7, which interact with p53 and Rb respectively, and cause immortalization of the cell.¹⁰⁸ Thus, the strain is now referred to as Human Newborn 18 E6E7 (HNB18E6E7). We have previously demonstrated that HNB18E6E7 interacts with fibrin clots in the exact manner as non-immortalized smooth muscle cells¹²⁵. Due to the fact that HNB18E6E7 human smooth muscle cells grow well in culture while most others do not, we preferentially utilize this cell strain. The cells were grown in Waymouth medium (GIBCO/BRL; Gaithersburg, MD) containing 0.23% sodium bicarbonate, 0.35 mg/ml L-glutamine, 1 mmol/L sodium pyruvate, and 0.01 mmol/L non-essential amino acids (GIBCO/BRL; Gaithersburg, MD). This basic medium was supplemented with 10% FCS (HyClone Laboratories, Inc.; Logan, UT). HNB18E6E7 smooth muscle cells were harvested at passages 23-27.

Whole Plasma, Gelatin Sepharose Treated Plasma, Recombinant Fibrinogen and Thrombin

Citrated human blood was centrifuged at 150 x g for 15 minutes at 20°C and the subsequent platelet-rich plasma was then centrifuged at 1400 x g for 20 minutes at 20°C. A portion of the platelet-poor plasma (called whole plasma) was stored at -70°C. The rest of the platelet-poor plasma was placed on a gelatin-sepharose 4B column (Pharmacia; Uppsala, Sweden) that was previously equilibrated with a buffer containing 0.05 mol/L Tris-HCL, pH 7.5, 5 mmol/L benzamidine and 0.02% sodium azide. The gelatin sepharose treated plasma was collected and the extent of fibronectin depletion determined by Western Blot analysis. In all cases, 80-90% of the fibronectin was removed from the plasma. The gelatin sepharose treated plasma was stored at -70°C. The production, purification and characterization of recombinant fibrinogen has already been described.^{111, 112} Human α -thrombin was purchased from Calbiochem (La Jolla, CA).

Purification of fibronectin from whole plasma

Fibronectin was isolated following a protocol published by Vuento and Vahe¹¹⁰. Platelet-poor plasma was placed on a gelatin-sepharose 4B column (Pharmacia; Uppsala, Sweden) that was previously equilibrated with a buffer containing 0.05 mol/L Tris-HCL, pH 7.5, 5 mmol/L benzamidine and 0.02% sodium azide. The gelatin-sepharose-fibronectin column was washed with a 1 mol/L NaCl, 0.05 mol/L Tris-HCL, pH 7.5 buffer followed by a wash with a buffer containing 0.2 mol/L arginine, 0.05 mol/L Tris-HCL, pH 7.5. Fibronectin was eluted from the column in fractions using a 1 mol/L arginine, 0.05 mol/L Tris-HCL, pH 7.5 buffer. The fibronectin was dialyzed overnight at 4°C in 0.05 mol/L Tris-HCL, pH 7.5. Fibronectin was concentrated using Centriprep concentrators (Amicon; Beverly, MA).

Coupling of Fibronectin to Affi-gel 10 column

4 mls of Affi-gel 10 agarose (Bio Rad; Hercules, CA) was washed in cold dH₂O twice. The slurry was resuspended in a buffer containing 0.1 mol/L MOPS pH 6.5, 80mmol/L CaCl₂. Human plasma fibronectin isolated from human blood and purchased from Sigma Chemical Co (St. Louis, MO) was added to the slurry. The mixture was rotated on a wheel for 4 hours at 4°C. 20 µl of 0.1 mmol/L ethanolamine, pH 8.0, was added to the slurry and the slurry incubated at 4°C for another hour. The slurry was loaded into a 10 ml column (Bio Rad; Hercules, CA), washed with cold dH₂O, washed with Pierce ImmunoPure Ag/Ab Gentle Elution Buffer (Rockford, IL) and then washed with Pierce ImmunoPure Ag/Ab Gentle Binding Buffer (Rockford, IL).

Purification of R790, anti-fibronectin polyclonal antibody

R790, a polyclonal anti-fibronectin antibody serum, was a gift from Dr. William Carter¹⁴². The serum was placed over a DEAE affi-gel blue-affinity chromatography column (Bio Rad; Hercules, CA) to remove any contaminating serum proteins such as albumin. Subsequently, the serum was placed on the affi-gel-10-fibronectin column described above. The serum and the subsequent flow-through were reloaded multiple times on the column to insure that all the anti-fibronectin antibody had attached.

The column was washed twice with 10 bed volumes of Pierce ImmunoPure Ag/Ab Gentle Binding Buffer and the antibody was eluted with Pierce ImmunoPure Ag/Ab Gentle Elution Buffer (Rockford, IL). The antibody was dialyzed overnight in a buffer containing 50 mmol/L Tris, pH 7.5, 100 mmol/L NaCl. The antibody was concentrated using Amicon Centriprep concentrators (Beverly, MA).

Flow Cytometry

Human newborn smooth muscle cell lines were harvested with trypsin-EDTA (GIBCO/BRL; Gaithersburg, MD), washed twice in PBS containing 0.5 mg/ml of Soybean Trypsin Inhibitor (Sigma Chemical Co; St. Louis, MO) and incubated on ice with the primary antibody (1:100 dilution) in 50 μ l of PBS containing 0.2% BSA (Sigma Chemical Co; St. Louis, MO) for 2 hours. The samples were subsequently washed three times with PBS containing 0.2% BSA, and incubated for 1 hour with either the phycoerythrin conjugated goat anti-mouse IgG secondary antibody (Biomeda; Foster City, CA) or the fluorescein isothiocyanate conjugated swine anti-rabbit IgG secondary antibody (Dako Corp.; Carpinteria, CA). Cells were washed in PBS and fixed using 1% paraformaldehyde. Peak Fluorescence Intensity was measured on a FACScan flow cytometer (Becton Dickinson Immunocytometry System; San Jose, CA).

Adhesion Assay

This assay has been previously described¹²⁵. Either whole plasma (1:5 dilution), gelatin sepharose treated plasma (1:5 dilution) or recombinant fibrinogen (0.25 mg/ml) were added to each well of a 96 well immunosorbant plastic plate (Nunc; Naperville, IL). An equal amount of thrombin (5 units/ml) was then added to each well and the plate was stored overnight at 4°C. The wells were washed with PBS, blocked with BSA-PBS for 1 hour at 37°C and pre-incubated with anti-human fibronectin antibodies for 15 minutes. The smooth muscle cells (50,000 cells/well) were pre-incubated for 15 minutes at room temperature with the indicated antibody in serum-free Waymouth medium containing 1 mg/ml of BSA, and then allowed to adhere to the substrate at 37°C/5% CO₂ for 1 hour. Non-adhering

cells were washed off using PBS and the adhering cells were fixed in 4% paraformaldehyde and stained with 4% paraformaldehyde containing 0.5% toluidine blue. The color was solubilized using 1% SDS and color intensity was measured using an ELISA reader set at 595nm¹¹³. All experiments were carried out independently at least twice with n=3 for each group.

Plasma Clot Contraction Assay

This assay is based on the one published by Ylanne *et al.* and has been previously described^{67, 125}. Cultured human newborn smooth muscle cell lines were harvested using trypsin-EDTA, washed twice in 0.5 mg/ml of soybean trypsin inhibitor (Sigma Chemical Co.; St. Louis, MO), and resuspended at a concentration of 10⁷ cells/ml in serum-free, Hepes (25 mmol/L) buffered-DMEM (GIBCO/BRL; Gaithersburg, MD). Human plasma (1:5 dilution), gelatin sepharose treated plasma (1:5 dilution) or recombinant fibrinogen (0.75 mg/ml) were added to the smooth muscle cells in a 6 mm X 50 mm pre-siliconized, autoclaved borosilicate test tube. Lastly, a mixture containing 28 mmol/L CaCl₂ and 5 units/ml of thrombin in serum-free, Hepes-DMEM was added to the test tube. The test tubes were incubated at 37°C with 5% CO₂. Pictures were taken with an Olympus OM2 camera using Kodak Ektachrome T64 slide film (Rochester, NY) and a Zuiko 50 mm f3.5 macro lens at t=0 and at time points specified in the figure legends. The slide was projected onto a piece of paper and the clot was traced. From the tracing, a two dimensional area was calculated to determine the percent of clot contraction using the following equation.

$$\% \text{ Clot Contraction (t= 10 hrs)} = 100 - \frac{[\text{area t=10 hrs}]}{\text{area at t=0}} \times 100$$

The mean and the standard error (SE) were calculated (Microsoft Excel; Redmond, WA). All experiments were carried out independently at least twice with n=3 for each group. One representative experiment is shown.

Enzyme Linked Immunosorbant Substrate Assay (ELISA)

Either whole plasma (1:5 dilution), gelatin sepharose treated plasma (1:5 dilution) or recombinant fibrinogen (0.25 mg/ml) were added to each well of a 96 well immunosorbant plastic plate (Nunc; Naperville, IL). An

equal amount of thrombin (5 units/ml) was then added to each well and the plate was stored overnight at 4°C. Wells were washed twice with PBS and blocked with PBS containing 2% BSA and 10% normal serum from horse (monoclonal antibody) or goat (polyclonal antibody) for 1 hour at room temperature. The wells were rinsed thrice with PBS and the primary antibodies (mAb 3E1 or polyclonal antibody R790) were added for 1 hour at room temperature. The antibody concentrations are stated in the figure and figure legends. The wells were rinsed thrice with PBS and the secondary antibody was added for 1 hour at room temperature. For biotinylated horse anti-mouse secondary antibody, a 1:200 dilution in PBS/2% BSA was used. For biotinylated goat anti-rabbit secondary antibody, a 1:1000 dilution in PBS/2% BSA was used. The wells were rinsed thrice with PBS. The avidin-biotin-peroxidase conjugate, from the Vectastain Elite ABC kit (Vector Laboratories, Inc.; Burlingame, CA), was diluted in PBS/2% BSA, added to the wells and left for 1 hour at room temperature. The wells were rinsed thrice with PBS. Chromogenic solution (30mg of o-phenylenediamine in 30 mls of 0.1 mol/L sodium citrate (pH 4.5) with 15 µl of 30% H₂O₂) was added to all wells. The color was allowed to develop and the reaction was stopped by the addition of 4.5 mol/L sulfuric acid. The color intensity was measured at 490nm.

Western Blot Analysis of Fibronectin Proteins

2X SDS sample buffer was added to either the supernatant or the fibrin/cell clot obtained from the contraction assay. The proteins were electrophoresed on a 5% SDS polyacrylamide gel and then transferred to PolyScreen PVDF transfer membranes (NEN Research Products; Boston, MA) using standard protein transfer techniques. The PVDF membrane was first probed with either a monoclonal anti-human fibronectin antibody or a polyclonal anti-fibronectin antibody followed by either a goat anti-mouse antibody or a goat anti-rabbit antibody at 1/100,000 dilution. The proteins were visualized using Renaissance Western Blot chemiluminescence Reagent (NEN Life Science; Boston, MA) and by exposing the membrane to autoradiography film.

Statistical Analysis

StatView 4.5 for MacIntosh (Abacus Concepts, Inc., Berkeley, CA) was used to calculate the p-values for all of the data. Briefly, the percent clot contraction (contraction assay) or the absorbance at 595nm (adhesion assay) was entered into the program. The mean, variance, standard deviation and standard error were calculated. If the variance between groups differed more than 20 fold, transformation analysis was performed on the raw data. In this paper, the function used for the contraction assays was the log (x) where x represents the % clot contraction. For the adhesion assays, the function 1/x was used to transform the data, where x represents absorbance at 595nm. Using either the transformed data or raw data, an F-test was performed as well as an ANOVA. If the ANOVA conveyed a p-value less than 0.05, than a Fisher's PLSD was calculated. A p-value less than 0.05 indicates statistical significance.

Results

Brefeldin A does not inhibit human smooth muscle cells contraction of a fibrin clot

Previously, we demonstrated that human smooth muscle cells can contract both a gelatin sepharose and a recombinant fibrin clot in the presence of cycloheximide¹²⁵. When we analyzed the supernatant from these clots through Western Blot analysis, we detected the presence of human fibronectin. This suggests that when human smooth muscle cells are put into these matrices, stores of fibronectin are released into the clot supernatant¹²⁵. To determine whether cellular fibronectin is interacting with $\alpha 5\beta 1$ on the human smooth muscle cells as well as interacting with fibrin, we treated the cells with Brefeldin A, an inhibitor of golgi transport^{143, 144}. When we placed the cells in a recombinant fibrin clot, we saw that at 0.1 μg and 1.0 μg Brefeldin A did not significantly affect the cell's ability to contract a fibrin clot (Figure 3.1A). When we repeated the experiment using whole plasma as our source of fibrinogen, as expected we also didn't see any effect (data not shown).

We manually separated the recombinant fibrin-smooth muscle cell clot from the surrounding supernatant and carried out a Western Blot analysis on both the recombinant fibrin-smooth muscle cell clot and the supernatant. We used an anti-fibronectin monoclonal antibody, clone 3E1, that is specific for human fibronectin and does not recognize bovine fibronectin. We did not find that human fibronectin is present in the supernatant in the Brefeldin A treated cells demonstrating that Brefeldin A did inhibit fibronectin release from the cell (Figure 3.1B). Thus if fibronectin is the ligand for $\alpha 5\beta 1$, these data suggest that the ligand is nascent fibronectin on the cell surface itself.

Fibronectin is present on the surface of human smooth muscle cells

To determine whether fibronectin is already present on the surface of these cells before the start of the experiment, we looked at surface expression of fibronectin through flow cytometry. We incubated the cells with either the anti-human fibronectin monoclonal antibody, 3E1, or the anti-fibronectin polyclonal antibody, R790. We detected the binding of these antibodies to the cell surface by using either a phycoerythrin conjugated anti-mouse secondary antibody or a fluorescein isothiocyanate conjugated anti-rabbit secondary antibody. We found both human fibronectin and non-human fibronectin present on the cell surface (Table 3.1).

To address the same question in another way, we tested whether human smooth muscle cells will adhere to R790 in a standard adhesion assay. If the cells are able to adhere to the antibody, this suggests that there must be fibronectin present on the cell surface. We plated 1, 5 and 10 $\mu\text{g}/\text{ml}$ of R790, rabbit polyclonal IgG antibody and human fibronectin in triplicates in a 96 well plate. We found that human smooth muscle cells will adhere to 5 and 10 $\mu\text{g}/\text{ml}$ of R790 and human fibronectin but not to the same concentrations of a non-specific rabbit polyclonal IgG (Figure 3.2). We also found that human smooth muscle cells will not adhere to a polyclonal anti-fibronectin antibody (data not shown). We repeated this experiment using the monoclonal antibody, 3E1, as a substrate and saw smooth muscle cell adhesion to this antibody (data not shown). These experiments suggest that on the surfaces of human smooth muscle cells fibronectin is present.

Fibronectin is important in human smooth muscle cell adhesion to fibrin

To determine if fibronectin is important in human smooth muscle cell adhesion to fibrin, we utilized the blocking polyclonal anti-fibronectin antibody, R790, in a standard adhesion assay. We first preincubated the smooth muscle cells with R790 for 15 minutes and then added them to recombinant fibrin, gelatin sepharose treated plasma clots and whole plasma clots. Adhesion was not affected by the antibody (data not shown). Next we preincubated the fibrin matrices with the antibody for 15 minutes and found that human smooth muscle cell adhesion to recombinant fibrin, gelatin sepharose treated plasma clots and whole plasma clots was significantly inhibited in the presence of R790 (p -value <0.05) (Figures 3.3 A, B, C). A rabbit polyclonal IgG antibody did not have an effect on smooth muscle adhesion to fibrin when compared with the untreated control. As expected, the polyclonal anti-fibronectin antibody R790 inhibited human smooth muscle cell adhesion to human fibronectin (10 $\mu\text{g/ml}$) (p -value <0.001) (Figure 3.4A) but did not inhibit cell adhesion to collagen (10 $\mu\text{g/ml}$) (Figure 3.4B). These data confirm that R790 specifically blocks cell adhesion to fibronectin and that matrix fibronectin is a component in human smooth muscle cell adhesion to fibrin clots.

Smooth muscle cell contraction of whole plasma clots utilize cell surface fibronectin

We next tested the role of fibronectin in human smooth muscle cell mediated contraction of whole plasma clots. We found that when R790 was preincubated with either the cells (100 $\mu\text{g/ml}$) (Figure 3.5A) or with both the cells and the matrix (50 $\mu\text{g/ml}$) (Figure 3.5C), smooth muscle cell contraction of whole plasma clots was significantly inhibited (p -value <0.001). However, when only the whole plasma was incubated with 100 $\mu\text{g/ml}$ R790, clot contraction was not affected (Figure 3.5B). Rabbit IgG antibody incubated with the cells alone, matrix alone or with both the cells and the matrix did not have a significant effect on whole plasma clot contraction by human smooth muscle cells. Therefore, fibronectin present on the cell surface seems to play a role than plasma fibronectin in smooth muscle cell contraction of whole plasma clots.

Smooth muscle cell contraction of gelatin sepharose treated plasma clots utilize cell surface fibronectin

We also tested whether cell surface fibronectin was important in smooth muscle contraction of gelatin sepharose treated plasma clots-clots that have been depleted of fibronectin. When R790 at 100 µg/ml was preincubated with smooth muscle cells (p-value<0.001) (Figure 3.6A), clot contraction was significantly inhibited. When 50 µg/ml of R790 was preincubated with both the cells and the gelatin sepharose treated plasma, clot contraction was inhibited 100% (p-value<0.001) (Figure 3.6C). Again, it seems that cell surface fibronectin plays a role in smooth muscle contraction of gelatin sepharose treated plasma clots.

Smooth muscle cell contraction of recombinant fibrin clots utilize a non-human fibronectin

Lastly we tested the effect of the anti-fibronectin polyclonal antibody R790 on smooth muscle cell contraction of recombinant fibrin clots. We found that smooth muscle cell contraction of recombinant fibrin clots was significantly inhibited when R790 was preincubated with recombinant fibrinogen, a situation different from what was found for whole plasma (p-value<0.05) (Figure 3.7A). When either smooth muscle cells alone or both the smooth muscle cells and the recombinant fibrinogen were preincubated with R790, there was a decrease in clot contraction, but this was not statistically significant. These results are surprising because recombinant fibrinogen should not have any fibronectin.

A non human fibronectin is a contaminating protein in recombinant fibrinogen

To determine what could be the protein in recombinant fibrinogen that is responsible for smooth muscle cell interaction with fibrin and is also the ligand for $\alpha 5\beta 1$, we tested for the presence of fibronectin in recombinant fibrinogen by using an ELISA assay. When we used the anti-human fibronectin monoclonal antibody, 3E1, at 1: 10,000 and 1: 100.000 dilution, we could not detect any significant amounts of human fibronectin in the recombinant fibrin clot (Figure 3.8A). However, when using whole plasma clots as a substrate, we detected the presence of human fibronectin as

expected (Figure 3.8B). In contrast, when we used the polyclonal anti-fibronectin antibody, R790, at 1 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$ on both substrates, we detected fibronectin in both sources of fibrinogen (Figure 3.9 A, B). In all cases, mIgG and rabbit IgG did not give a positive result. Interestingly, when we looked for fibronectin in the gelatin sepharose treated plasma clots, R790 also was positive in the ELISA assay (data not shown). These data suggest that both human and non-human fibronectins are present in both gelatin sepharose treated plasma and recombinant fibrinogen. The human fibronectin could be smaller fragments of fibronectin that are not detected by the monoclonal antibody, 3E1.

To determine if there are smaller fragments of fibronectin present, we carried out a Western Blot Analysis on whole plasma, gelatin sepharose treated plasma and recombinant fibrinogen. When using 3E1 as the primary antibody, we found that in whole plasma, there were both high and low molecular weight human fibronectin present (Figure 3.10A, Lane 3). In gelatin sepharose treated plasma, there was only low molecular weight human fibronectin seen (Figure 3.10A, Lane 2). In recombinant fibrinogen, there was no human fibronectin in the sample (Figure 3.10, Lane 4). However, when we used R790 as the primary antibody, we found that in all three samples, fibronectin was present (Figure 3.10B, Lanes 1-4). We conclude that there are human fibronectin fragments in the gelatin sepharose treated plasma and that there is a non human fibronectin contaminating the recombinant fibrinogen. Because the recombinant fibrinogen is harvested from chinese hamster ovary cells that express the proteins, we believe that the fibronectin is hamster in origin.

Discussion

We previously demonstrated that human smooth muscle cells interact with fibrin through the integrin $\alpha 5\beta 1$ ¹²⁵. This integrin has always been considered to be a fibronectin receptor; however, recently Suehiro *et al.* demonstrated that in endothelial cells, it can be a receptor for fibrinogen in the presence of manganese⁸³. In the work presented here, we further

characterize smooth muscle interaction with fibrin by exploring the role of fibronectin in this interaction. To do this, we utilized two different types of anti-fibronectin antibodies, 3E1 and R790. 3E1 is a non-blocking, monoclonal anti-human fibronectin antibody that does not cross react with bovine fibronectin⁹⁵. From our data, it seems to recognize high molecular weight fibronectin-probably either dimer or monomer fibronectin. The other antibody is a blocking, polyclonal anti-fibronectin antibody that recognizes both the high molecular weight as well as the lower molecular weight fragments of fibronectin. This antibody also recognizes other species of fibronectin such as bovine fibronectin (W. Carter, unpublished data, 1998).

We first determined that actively secreted fibronectin is not critical for smooth muscle cell contraction of recombinant fibrin clots. We treated the cells with Brefeldin A, an inhibitor of the Golgi exocytosis pathway, and found that smooth muscle cells were able to contract a recombinant fibrin clot (Figure 3.1). Next we used flow cytometry analysis and a standard adhesion assay to determine if fibronectin was present on the cell surface after harvesting. By flow cytometry, we saw that both 3E1 and R790 recognized fibronectin on the surface of the cell. However, R790 had a peak fluorescent intensity 2.5 times greater than 3E1 (Table 3.1). This suggests that there are either fragments of fibronectin on the cell surface or that there is a non-human fibronectin, such as bovine fibronectin, present. These fibronectin fragments could be surface fibronectin that has been cleaved by trypsin during cell harvesting. When we looked at the ability of human smooth muscle cells to adhere to antibodies coated on plastic, we saw that the cells were able to bind to the anti-fibronectin antibodies but not to the non-immune mIgG or rabbit IgG antibodies. This suggests that there is an epitope on the cell surface that is recognized by the anti-fibronectin antibodies. From these data we conclude that fibronectin is present on the cell surface after cells are harvested.

We next looked at smooth muscle cell adhesion to three sources of fibrinogen: whole plasma, gelatin sepharose treated plasma and recombinant fibrinogen. When we pre-treated the cells with R790, cell adhesion to these matrices was not inhibited. However when we pretreated the matrices with R790 and then added the human smooth muscle cells to

the wells, we found that smooth muscle cell adhesion was greatly reduced in all three cases (Figure 3.3). Inhibition of smooth muscle cell adhesion to recombinant fibrin was surprising because we assumed that there weren't any other plasma proteins besides fibrinogen present. Interestingly, inhibition of cell adhesion to recombinant fibrin by R790 showed a dose dependent profile, while in both whole plasma clots and gelatin sepharose treated plasma clots, the degree of inhibition was higher and saturated at the initial concentration. Assuming that all the HNB18E6E7 smooth muscle cells are similar in regards to the amount of fibronectin on their surfaces, this could be due to (1) differences in the amount of fibronectin in the matrices, (2) differences in the amounts of high and low molecular weight fibronectin and (3) differences in the affinity of R790 for the different forms of fibronectin. These data suggest that smooth muscle cells adhesion to fibrin clots is mediated by fibronectin that is found in the matrices and that non-human fibronectin may be present in recombinant fibrinogen.

We also wanted to know whether smooth muscle cell mediated fibrin clot contraction relied on fibronectin. In these experiments we used all three sources of fibrinogen and pre-incubated either the cells alone, fibrinogen alone or both the cells and the fibrinogen with R790. When using whole plasma as our source of fibrinogen, clot contraction was inhibited when we incubated the cells with R790 while incubating only the matrix did not have an effect. When we incubated both the cells and the matrix (cells/matrix) with R790, contraction of whole plasma clots were inhibited. When we looked at smooth muscle cell contraction of gelatin sepharose plasma clots, incubation of both the cells and the cells/matrix with R790 inhibited clot contraction. This suggests that cell surface fibronectin is more important than soluble fibronectin for contraction of plasma clots. However, when we carried out the same experiments but used recombinant fibrin, clot contraction was only statistically inhibited if we only pre-incubated the matrix suggesting a greater role for the non-human fibronectin than the cell surface fibronectin in recombinant fibrin clot contraction by smooth muscle cells. These data indicated that the role of fibronectin in smooth muscle cell interaction with fibrin depends on the source of fibronectin.

This was confirmed when we looked at the forms of fibronectin in whole plasma vs. recombinant fibrinogen. By both ELISA and western blot analysis using the two antibodies, which differ in their specificity for human fibronectin, we found that in recombinant fibrinogen, there are high and low molecular weight fibronectins present that are not recognized by the human specific anti-fibronectin antibody, 3E1, but are recognized by the anti-fibronectin antibody, R790 (Figures 3.9, 3.10). We also found out by Western Blot analysis that fibronectin depleted plasma is only depleted of the high molecular weight form of fibronectin (Figures 3.10A, B). There are plenty of fibronectin fragments present and these fragments probably do play a role in smooth muscle cell interaction with fibrin (Figure 3.10).

The solubility of the fibronectin in each assay may be the reason why there are such diverse results between the different substrates and the different assays, adhesion vs. contraction. In the adhesion assay, the fibronectin is insoluble and easily recognized by the cell. This may be the reason why blocking the matrix with the antibody R790 was more effective in inhibiting adhesion than blocking the smooth muscle cells. While in the contraction assays, the fibronectin is mainly soluble though it is possible that cross-linking by Factor XIIIa will render it insoluble within the fibrin matrix and accessible to the cell's integrin receptors. However, because pre-incubation of the plasma did not inhibit contraction of plasma clots, only a small percentage of the fibronectin may be cross-linked. It must also be taken into consideration that the smooth muscle cells themselves are probably secreting fibronectin and this fibronectin is deposited on the cell surface or in the supernatant. The data supports the idea that during contraction of whole plasma and gelatin sepharose treated plasma clots, cell surface fibronectin mediates smooth muscle cell interaction with fibrin.

The data is a little more complicated with recombinant fibrinogen. Under these conditions there are two sources of fibronectin, smooth muscle cells and chinese hamster ovary fibroblasts. During secretion of the recombinant fibrinogen from the hamster fibroblasts cells, fibronectin may be cross-linked to the fibrinogen by transglutaminases. This hamster fibronectin would then be insoluble when the fibrin clot forms and more easily accessible to the cells than the fibronectin secreted in the supernatant. However, smooth muscle cells are probably also secreting fibronectin onto

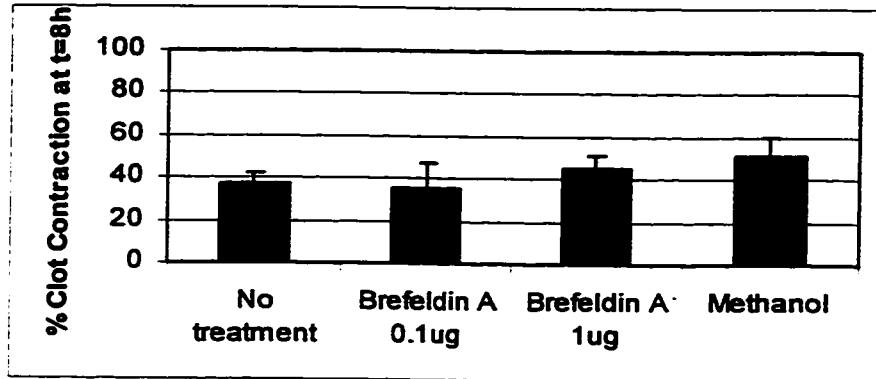
the surface of the cells, the Brefeldin A data imply that this fibronectin is also playing a role in smooth muscle cell interaction with fibrin. In our contraction data, we saw that when we incubated the cells, the matrix and both the cells and matrix with R790, in all cases we saw inhibition of clot contraction. Only in the case where the matrix alone was incubated with the antibody did we see a statistical significance. This suggest that perhaps the cell surface fibronectin is important as well though not as important as the hamster fibronectin that is already present in the matrix.

In conclusion we have demonstrated that smooth muscle cells interaction with fibrin is mediated by the extracellular matrix protein fibronectin. We have shown that both cell surface and matrix fibronectins can function in this interaction, depending upon the solubility of the fibronectin. Other groups have also shown that fibronectin is able to interact with fibrinogen and that this matrix is more conducive for cell interaction than fibrin alone^{140, 141}. However, in previous publications where others have characterized fibroblast, melanoma, endothelial and CHO cells interaction with fibrin, the fibronectin component wasn't revealed^{67, 69, 116, 145}. This may be to due to the integrin surface expression on the cells. In human smooth muscle cells, $\alpha v \beta 3$, a known fibrin receptor, is not highly expressed while in these other cell types it is highly expressed. On our cells and on other cells, the fibronectin receptor, $\alpha 5 \beta 1$, is also highly expressed. This difference in integrin expression may direct how the cells are interacting with the matrix. In our case, the low expression of the fibrin receptor, $\alpha v \beta 3$, forces the cells to search for other matrix proteins since $\alpha v \beta 3$ is rarely found in intimal smooth muscle cells, even in fibrin in plaques. Even though $\alpha 5 \beta 1$ interaction with fibronectin is found in many cell types and many biological situations, the role of fibronectin as a mediator between smooth muscle cell $\alpha 5 \beta 1$ and fibrin may be important in atherosclerosis progression.

Figure 3.1: Effect of Brefeldin A on recombinant clot contraction by HNB18E6E7.

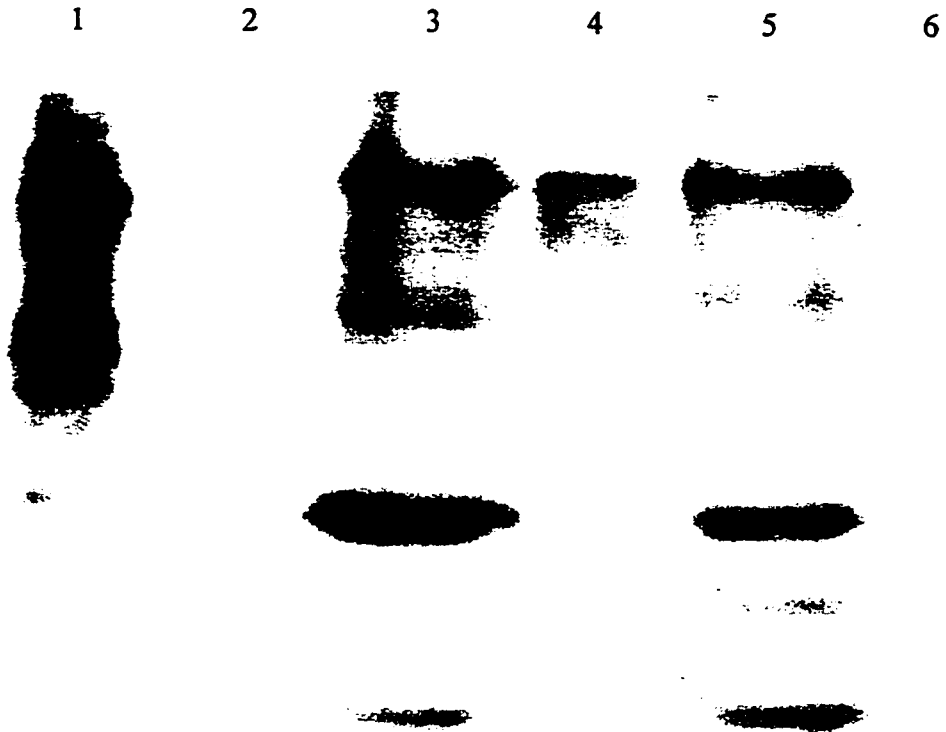
Human smooth muscle cells were untreated or treated with either (A) 0.1 μg or 1 μg of Brefeldin A for 30 minutes at room temperature. Recombinant fibrinogen and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 8 hours. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3). (B) Western Blot Analysis to determine the presence of fibronectin in the supernatant or in the fibrin/smooth muscle cell clot in the presence and absence of Brefeldin A. Either the supernatant or the clot was removed from the experiment in figure 3.1A. The samples were electrophoresed on a 5% SDS-PAGE, transferred to a membrane and probed with an anti-human fibronectin antibody and a goat-anti-mouse secondary antibody.

A



B

1. Human Fibronectin
2. Recombinant Fibrin only
3. Cell lysate (No treatment)
4. Supernatant (No treatment)
5. Cell lysate (Brefeldin A 0.1ug)
6. Supernatant (Brefeldin A 0.1ug)



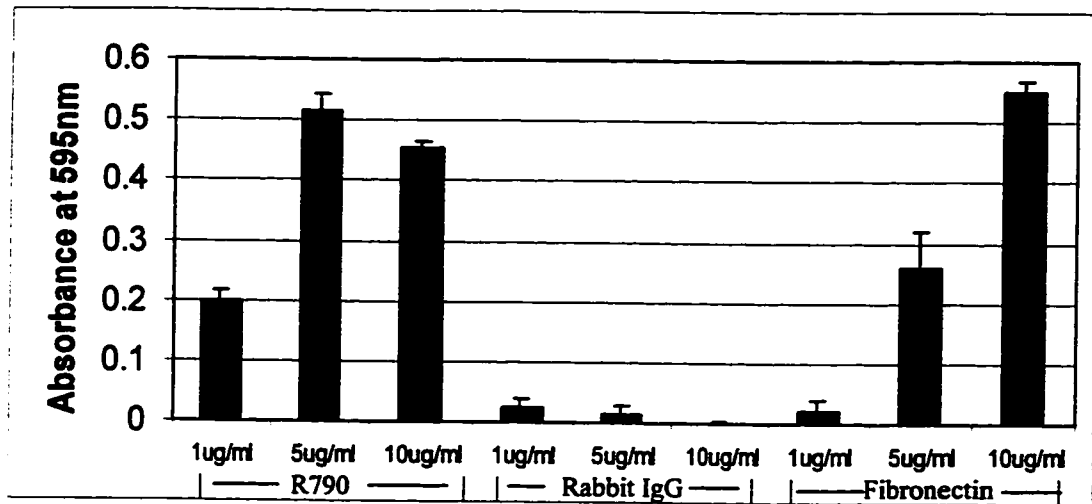


Figure 3.2: Adhesion of HNB18E6E7 smooth muscle cells to an anti-fibronectin polyclonal antibody, R790, Rabbit IgG and human fibronectin.

R790, Rabbit IgG and human fibronectin at 1 µg/ml, 5 µg/ml and 10 µg/ml were coated onto a 96 well plate and stored overnight for 24 hrs at 4°C. Human smooth cells were allowed to adhere to the substrate for 1 hour at 37°C, and non-adhering cells were washed off with PBS. The adhering cells were fixed with 4% paraformaldehyde and stained with toluidine blue. The color intensity was measured by an ELISA reader at 595nm. Values represent the mean ± standard error (n=3).

Figure 3.3: Inhibiting cell adhesion assay using R790

(A) Inhibiting adhesion of HNB18E6E7 smooth muscle cells to recombinant fibrin using R790, an anti-fibronectin polyclonal antibody. Recombinant fibrin and thrombin were coated onto a 96 well plate overnight for 24 hrs at 4°C. The recombinant fibrin matrix was pretreated with either 10 µg/ml, 25 µg/ml or 50 µg/ml of R790 or rabbit IgG. Human smooth muscle cells were allowed to adhere to the substrate for 1 hour at 37°C, and non-adhering cells were washed off with PBS. The adhering cells were fixed with 4% paraformaldehyde and stained with toluidine blue. The color intensity was measured by an ELISA reader at 595nm. Values represent the mean divided by the mean of the No Ab control ± standard error (n=3). **(B)** Inhibiting adhesion of HNB18E6E7 smooth muscle cells to gelatin sepharose treated plasma clots using R790, an anti-fibronectin polyclonal antibody. The procedure is the same as in 3.3A except that gelatin sepharose treated plasma clots were pretreated with either 10 µg/ml, 25 µg/ml or 50 µg/ml of R790 or rabbit IgG. Values represent the mean divided by the mean of the No Ab control ± standard error (n=3). **(C)** Inhibiting adhesion of HNB18E6E7 smooth muscle cells to whole plasma clots using R790, an anti-fibronectin polyclonal antibody. The procedure is the same as in 3.3A except that whole plasma clots were pretreated with either 10 µg/ml, 25 µg/ml or 50 µg/ml of R790 or rabbit IgG. Values represent the mean divided by the mean of the No Ab control ± standard error (n=3).

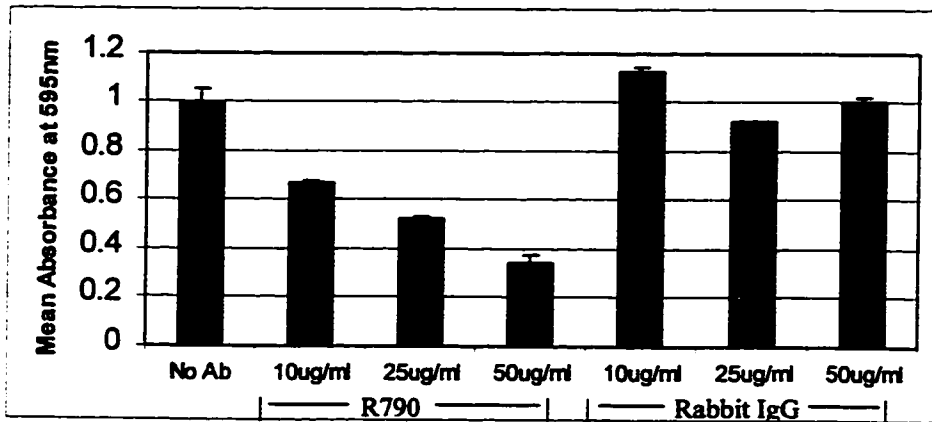
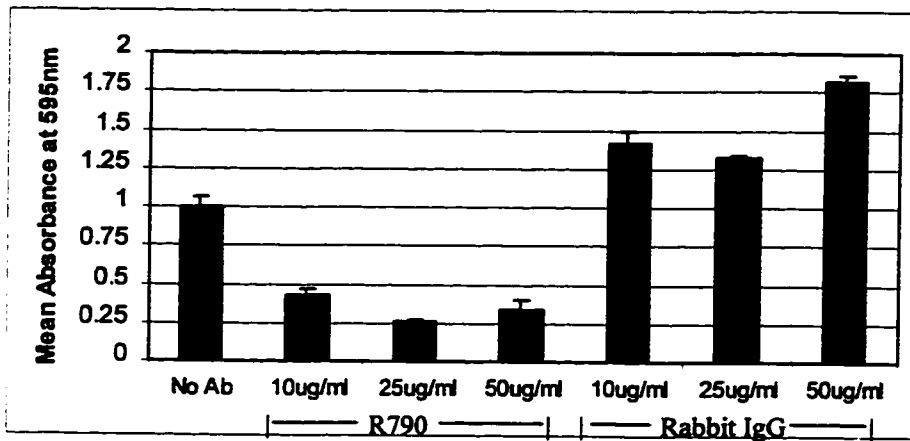
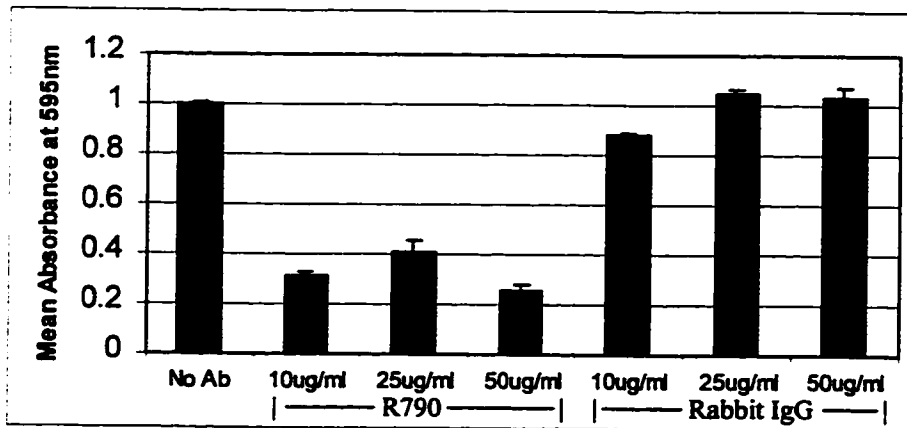
A**B****C**

Figure 3.4: Inhibiting adhesion of HNB18E6E7 smooth muscle cells to human fibronectin and collagen using R790, an anti-fibronectin polyclonal antibody

(A) Human fibronectin at 10 $\mu\text{g/ml}$ was coated onto a 96 well plate overnight for 24 hrs at 4°C. The human fibronectin was pretreated with either 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$ of R790 or rabbit IgG. Human smooth muscle cells were allowed to adhere to the substrate for 1 hour at 37°C, and non-adhering cells were washed off with PBS. The adhering cells were fixed with 4% paraformaldehyde and stained with toluidine blue. The color intensity was measured by an ELISA reader at 595nm. Values represent the mean divided by the mean of the No Ab control \pm standard error (n=3). **(B)** The procedure is the same as in 3.3A except that collagen at 10 $\mu\text{g/ml}$ was pretreated with either 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$ of R790 or rabbit IgG. Values represent the mean divided by the mean of the No Ab control \pm standard error (n=3).

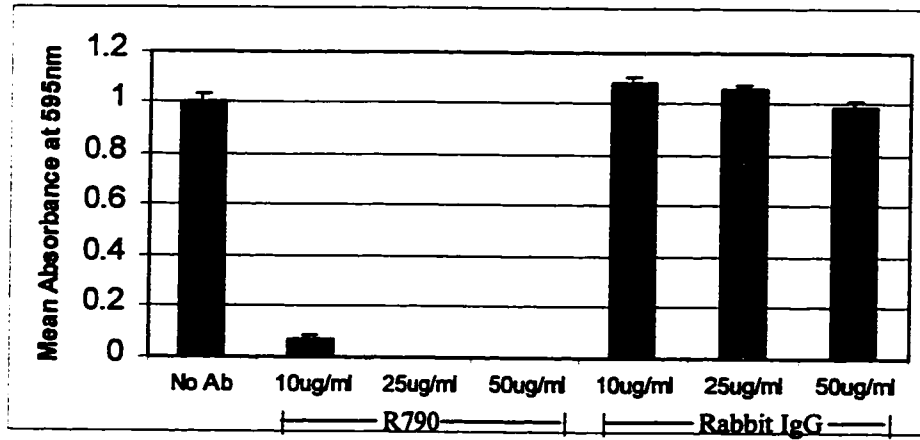
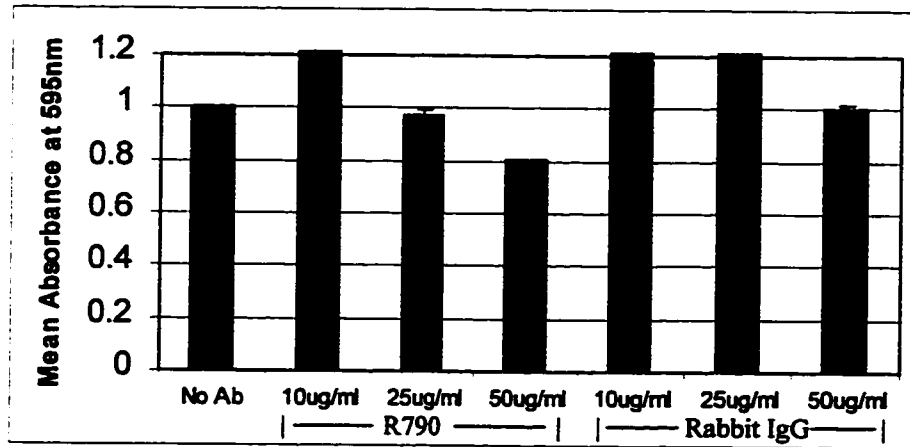
A**B**

Figure 3.5: Effect of R790, anti-fibronectin polyclonal antibody, on whole human plasma clot contraction by HNB18E6E7.

(A) Human smooth muscle cells were untreated or treated with either 100 $\mu\text{g}/\text{ml}$ of R790 or Rabbit IgG **(B)** Whole plasma was untreated or treated with 100 $\mu\text{g}/\text{ml}$ of R790 or Rabbit IgG **(C)** Human smooth muscle cells and whole plasma were untreated or treated with either 100 $\mu\text{g}/\text{ml}$ of R790 or Rabbit IgG Whole human plasma and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 10 hours. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3).

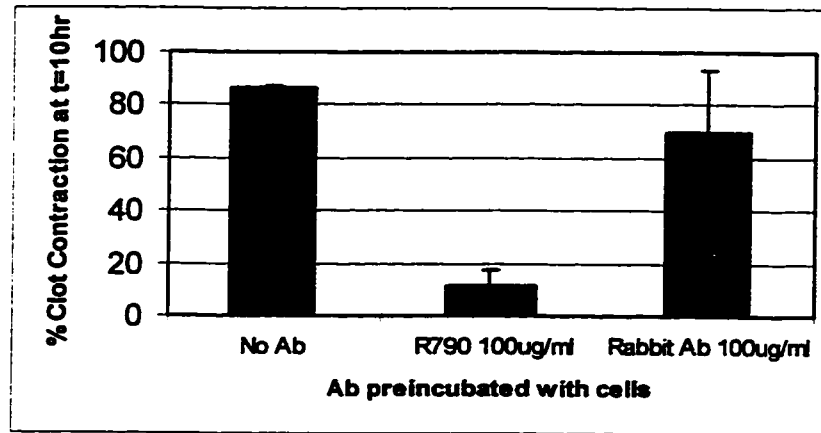
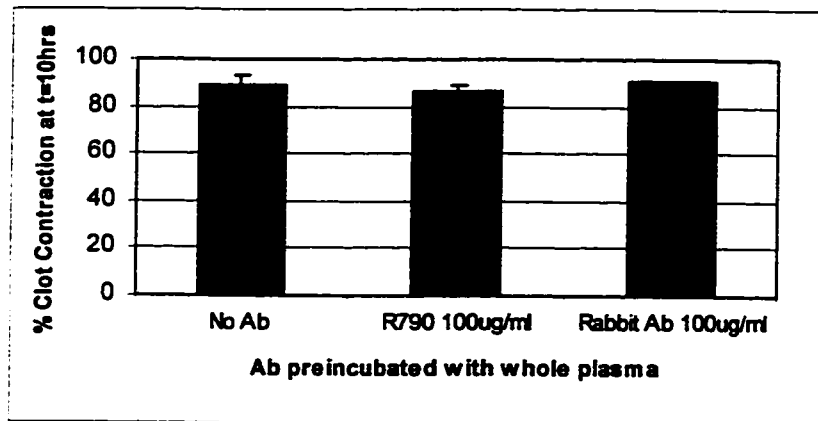
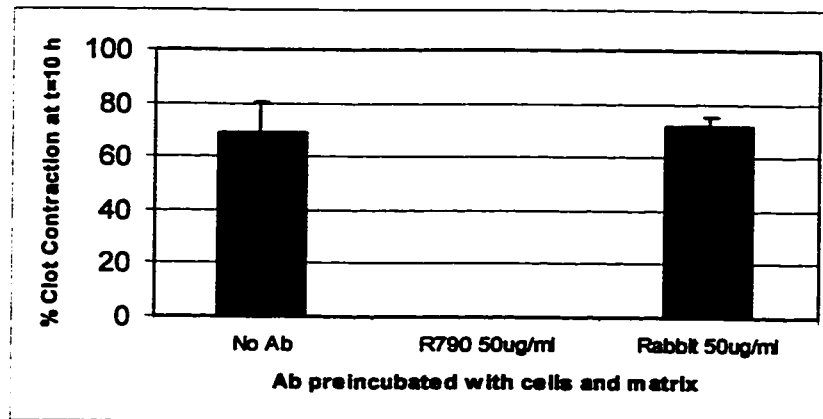
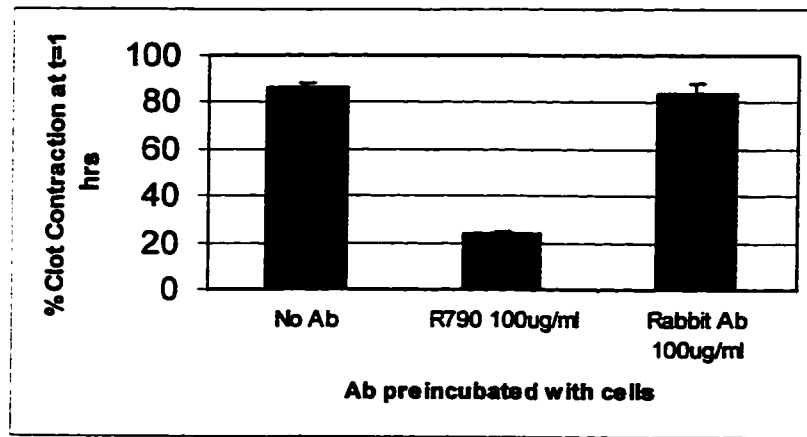
A**B****C**

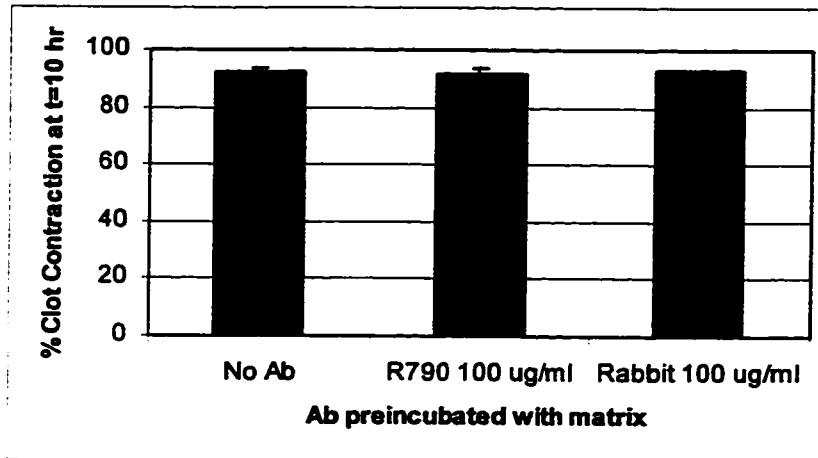
Figure 3.6: Effect of R790, anti-fibronectin polyclonal antibody, on gelatin sepharose treated human plasma clot contraction by HNB18E6E7.

(A) Human smooth muscle cells were untreated or treated with either 100 $\mu\text{g}/\text{ml}$ of R790 or Rabbit IgG **(B)** Gelatin sepharose treated human plasma was untreated or treated with 100 $\mu\text{g}/\text{ml}$ of R790 or Rabbit IgG **(C)** Human smooth muscle cells and gelatin sepharose treated human plasma were untreated or treated with either 100 $\mu\text{g}/\text{ml}$ of R790 or Rabbit IgG Gelatin sepharose treated human plasma and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 10 hours. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3).

A



B



C

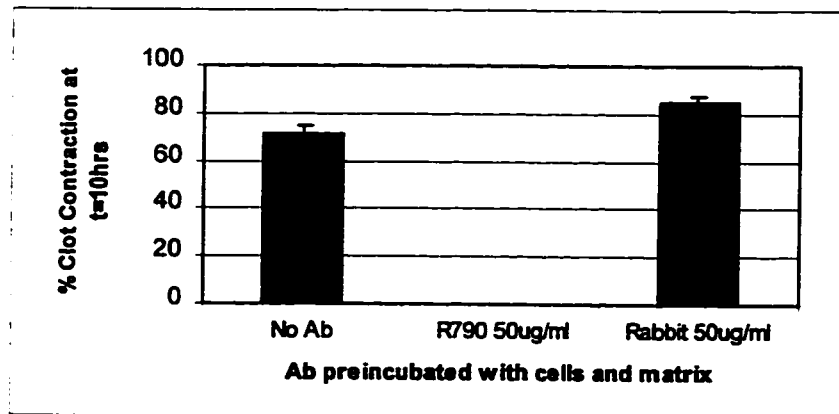
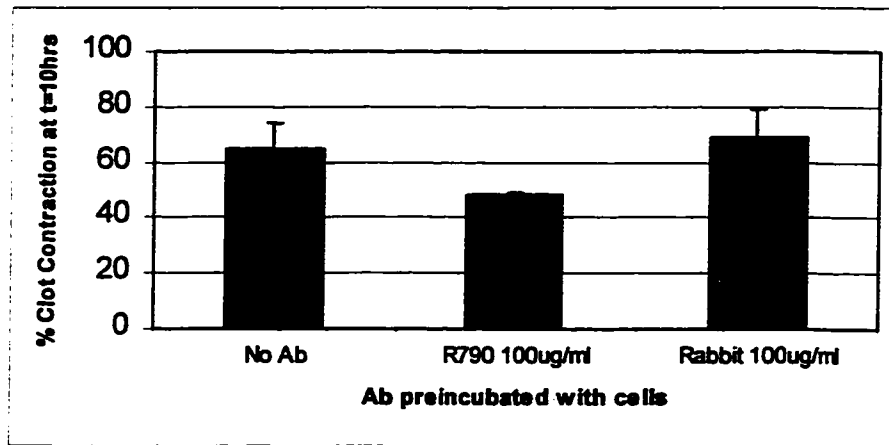
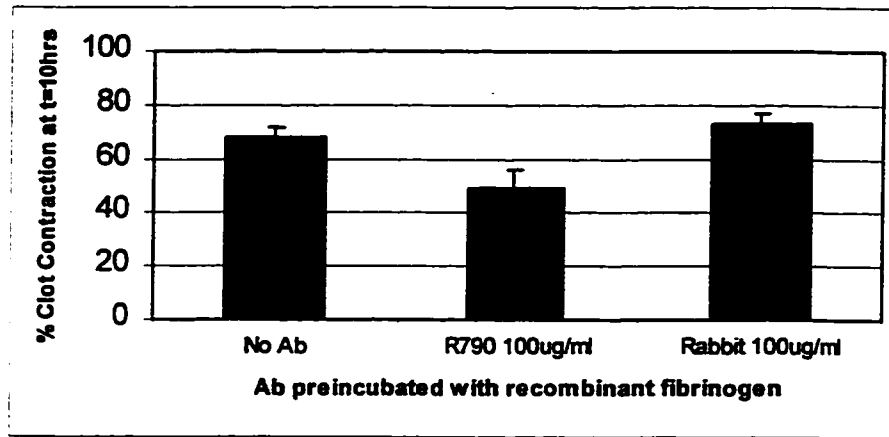
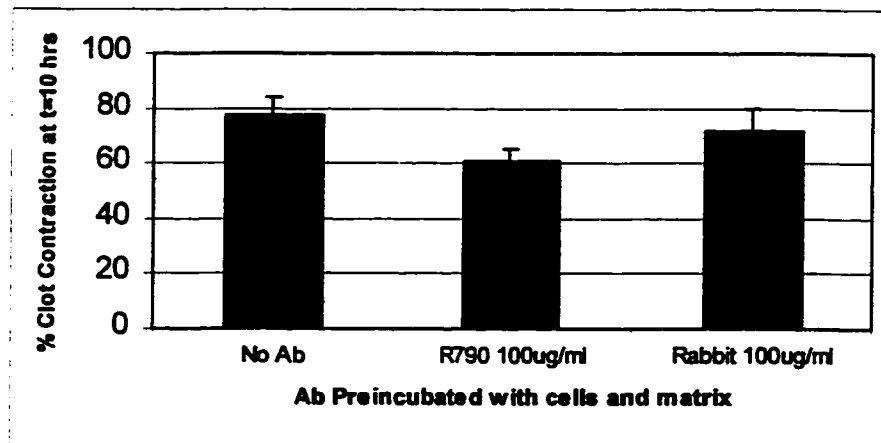


Figure 3.7: Effect of R790, anti-fibronectin polyclonal antibody, on recombinant human fibrin clot contraction by HNB18E6E7.

(A) Human smooth muscle cells were untreated or treated with either 100 $\mu\text{g}/\text{ml}$ of R790 or Rabbit IgG (B) Recombinant human fibrinogen was untreated or treated with 100 $\mu\text{g}/\text{ml}$ of R790 or Rabbit IgG (C) Human smooth muscle cells and recombinant human fibrinogen were untreated or treated with either 100 $\mu\text{g}/\text{ml}$ of R790 or Rabbit IgG Recombinant human fibrinogen and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 10 hours. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3).

A**B****C**

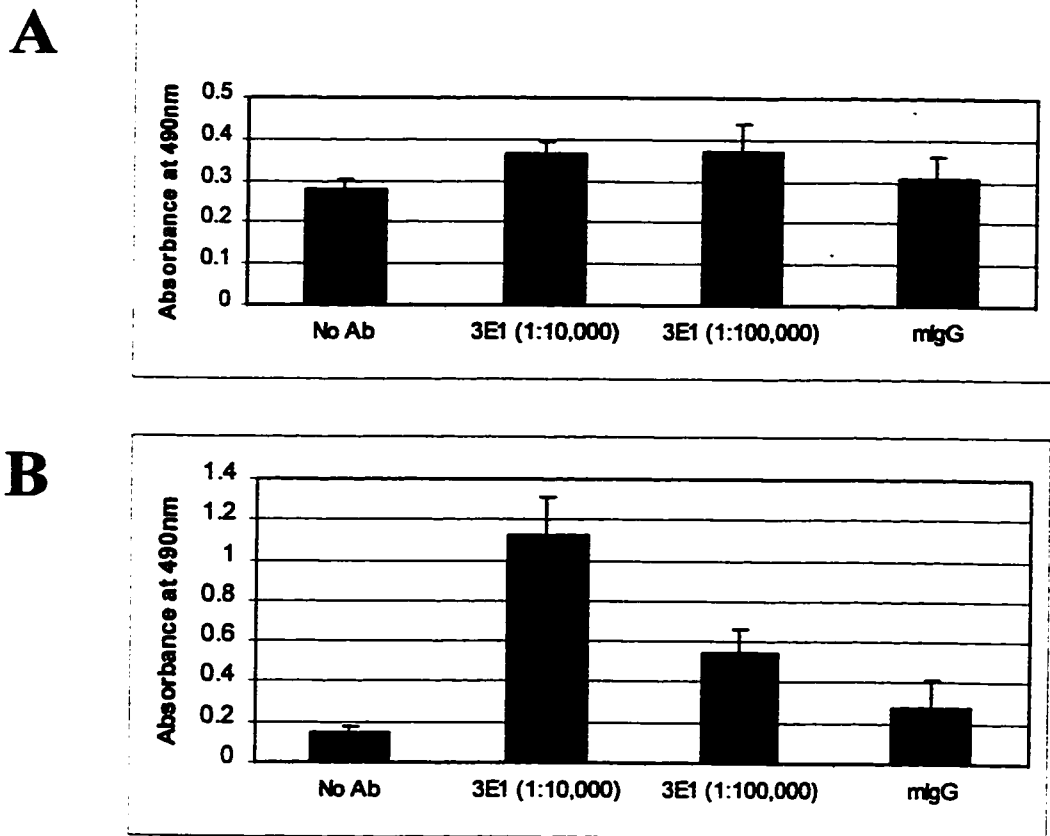


Figure 3.8: ELISA assay to detect human fibronectin in recombinant human fibrinogen and human whole plasma.

(A) Recombinant human fibrinogen at 0.25 mg/ml or (B) human whole plasma were placed in the wells of a 96 well plate overnight. Monoclonal anti-human fibronectin antibody 3E1 was added to the plates at 1: 10,000 and 1:100,000 dilution. MIgG antibody was also added to the wells. After washing with PBS, the biotinylated horse anti-mouse secondary antibody was added to the wells. Finally avidin conjugated peroxidase was added and color intensity was measured at 490nm. Values represent mean \pm standard error (n=3).

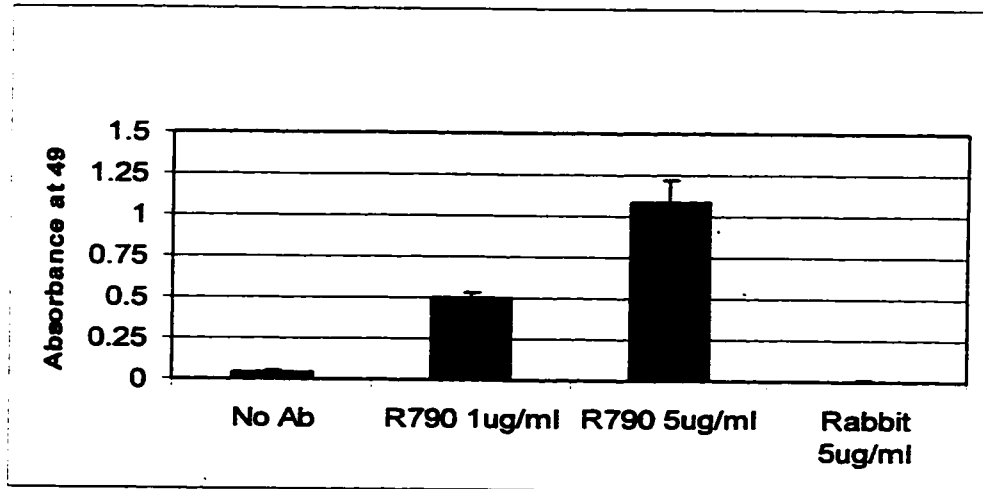
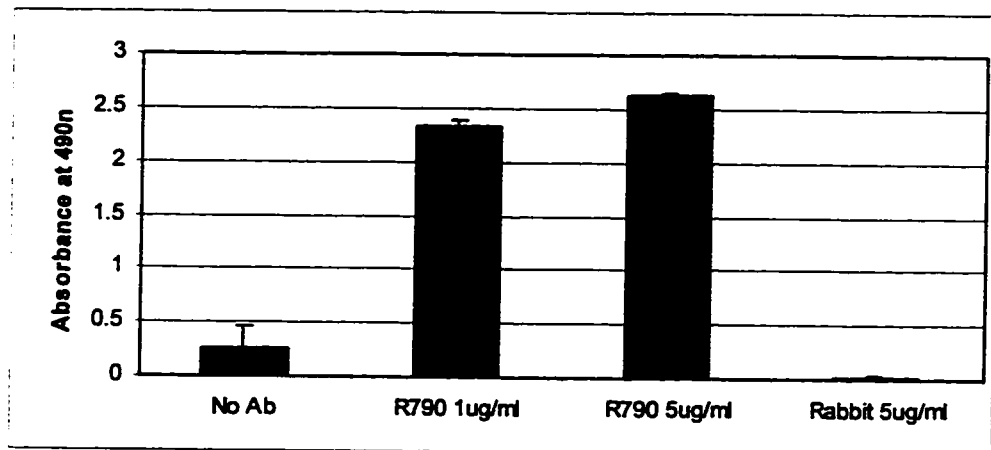
A**B**

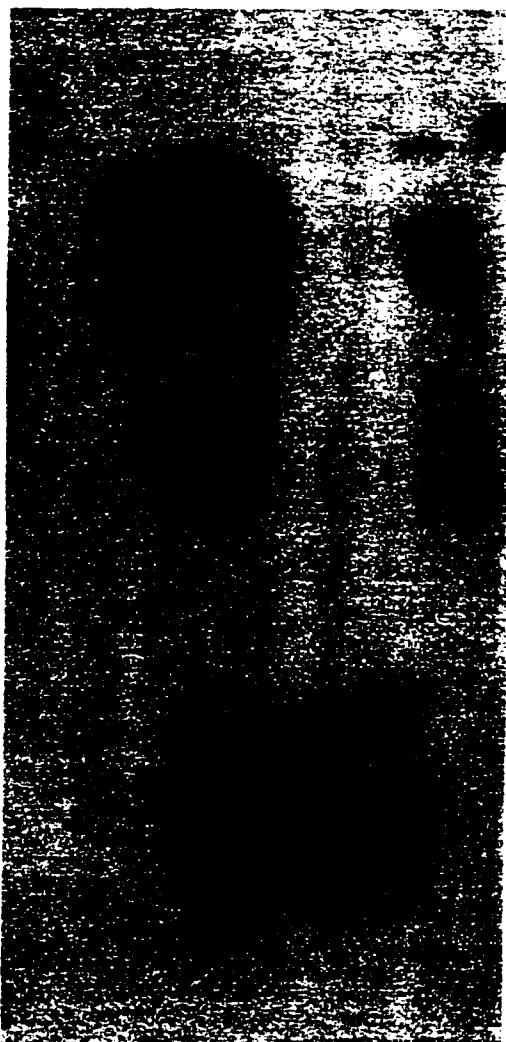
Figure 3.9: ELISA assay to detect fibronectin in recombinant human fibrinogen and human whole plasma.

(A) Recombinant human fibrinogen at 0.25 mg/ml or **(B)** human whole plasma were placed in the wells of a 96 well plate overnight. This assay is similar to what was described in Figure 3.7 except polyclonal anti-fibronectin antibody, R790, was added to the plates at 1 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$. Rabbit IgG was also added to the wells at 5 $\mu\text{g}/\text{ml}$. After washing with PBS, the biotinylated goat anti-rabbit secondary antibody was added to the wells. Finally avidin conjugated peroxidase was added and color intensity was measured at 490nm. Values represent mean \pm standard error ($n=3$).

Figure 3.10: Western Blot Analysis to determine the presence of fibronectin in whole plasma, gelatin sepharose treated plasma and recombinant fibrinogen.

Samples of whole plasma gelatin sepharose treated plasma and recombinant fibrinogen were electrophoresed through a 5 % SDS polyacrylamide gel. The proteins were transferred to a membrane and probed with either (A) monoclonal anti-human fibronectin antibody, 3E1, at 1:10,000 dilution or (B) polyclonal ant-fibronectin antibody, R790, at 1:10,000 dilution

B



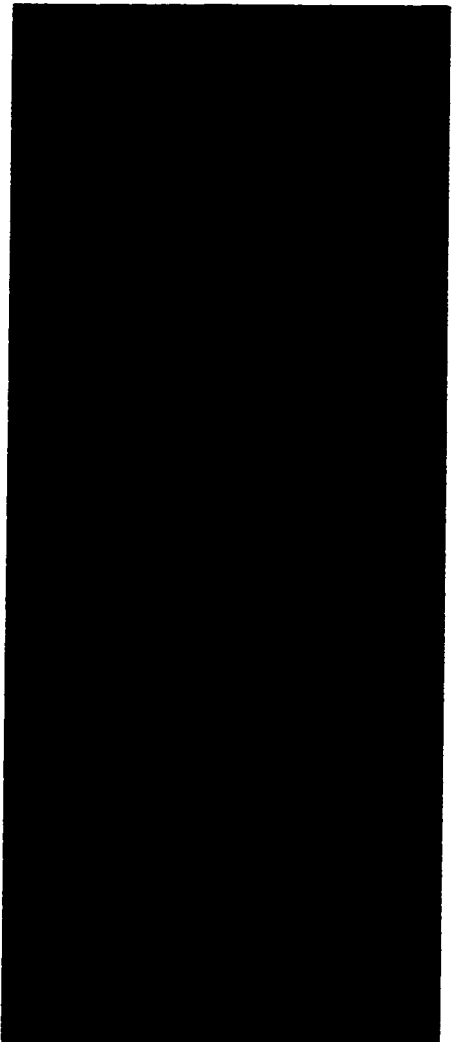
Human fibronectin

Gelatin sepharose plasma

Whole plasma

Recombinant fibrinogen

A



Recombinant fibrinogen

Whole plasma

Gelatin sepharose plasma

Human fibronectin

Table 3.1: Expression of fibronectin on the surface of HNB18E6E78

| Antibody | Peak Fluorescent Intensity |
|-----------------|-----------------------------------|
| mIgG | 1.0 |
| 3E1 | 6.38 |
| P4C10 | 69.6 |
| rabbit IgG | 1.0 |
| R790 | 16.79 |

**Chapter 4: Kistrin Inhibits Human Smooth Muscle Cell
Interaction with Fibrin**

**This work was submitted to *Circulation Research* by Karen O Yee, Yuji Ikari,
Sarah Bodary and Stephen M. Schwartz.**

Introduction to Chapter 4

Until recently the conventional wisdom has been that vessels narrow in atherosclerosis or restenosis because of an increase in intima mass¹. However morphometric studies of autopsy tissues as well as intravascular ultrasound studies of restenotic vessels have shown a lack of correlation between lumen size and intimal mass^{2, 3, 74}. This has led to the somewhat vague notion that the loss of lumen is attributed to "remodeling" which could be defined as a redistribution of mass or a morphological constriction^{2, 146}. In an effort to explore possible mechanisms for narrowing after injury, we focused on the possible role of intramural fibrin. We chose fibrin because many groups have demonstrated the presence of fibrinogen and fibrin in the arterial wall and in the atherosclerotic plaque^{44, 47-50}. Furthermore, others have shown that fibrinogen blood indexes indicate the extent of coronary artery disease, carotid artery disease and peripheral arterial disease^{51, 147}. We believe that cell-fibrin interactions could contribute to lumen narrowing during atherosclerosis.

Recently, Courtman *et al.* demonstrated that fibrin has a role in vessel narrowing after angioplasty. By introducing a factor VIIa inhibitor, VIIai, fibrin formation and lumen narrowing were prevented in a rabbit model of restenosis³⁷. Specifically, at 6 weeks after the second balloon injury, the lumen of the untreated vessel was smaller than that of the treated vessel without a difference in media and adventia thickness. This suggested that fibrin was important in the remodeling process. In another study, Geary *et al.* showed that wound healing could cause lumen narrowing after arterial reconstruction³⁹. They performed experimental angioplasty on monkeys that were fed an atherogenic diet for three to five years. They found that lumen narrowing was caused by a decrease in artery wall diameter. They also characterized the matrix and integrin expression patterns on these vessels and concluded that they were similar to those found in healing wounds. In fact, fibrin was detected in these vessels. Therefore wound contraction may play a role in lumen narrowing in vessels that have undergone angioplasty. These two studies highlight the possible importance of both fibrin, a wound matrix protein, and wound contraction as causes of vessel narrowing after injury.

A number of drugs, such as Reopro, have been developed in an attempt to prevent this process. Reopro is a humanized antibody against both the $\alpha v \beta 3$ and $\alpha II b \beta 3$ integrins^{100, 114}. In clinical trials this product has decreased significantly the incidence of clinical events after angioplasty. This prevention has also been shown to be long term, lasting up to 3 years post angioplasty¹⁴⁸. The long term effect of Reopro suggests that smooth muscle cells may have a role in vessel remodeling since platelets will not be present in the wound area 3 years after injury¹⁴⁹.

Other groups have tried to prevent lumen narrowing using a different approach. They used integrin antagonists to prevent intimal thickening in models of atherosclerosis. Specifically, they have used peptide antagonists such as linear and cyclic RGD peptides. Matsuno *et al.* demonstrated in hamsters that a cyclic RGD peptide, G4120, effectively prevented neointima formation and was more effective in combination with quinapril, an angiotensin converting enzyme inhibitor^{150, 151}. Choi *et al.* showed that in a rabbit carotid balloon injury model, a cyclic peptide, penRGD, inhibited neointima formation with a difference in the intima:media ratio¹⁵². In all of these studies, the authors hypothesized that smooth muscle cell migration and proliferation were inhibited by these peptides. The target of the peptide was assumed to be the integrin $\alpha v \beta 3$, consistent with the evidence that this integrin plays a critical role in migration on vitronectin and fibrin by many cell types¹⁵³⁻¹⁵⁵

Previously we have shown that human smooth muscle cell interaction with fibrin could be prevented by using antibodies against fibronectin and the $\alpha 5 \beta 1$ integrin^{125, 156}. Integrins are transmembrane glycoproteins that form a heterodimer composed of an alpha and a beta chain⁵⁸. The family of integrins are composed of 15 alpha chains and 8 beta chains¹⁵⁷. Heterodimers of these alpha and beta chains can be formed to confer specific binding to extracellular matrix proteins such as collagen, laminin, vitronectin, fibronectin and fibrin(ogen). The integrins of interest in our studies of atherosclerosis and luminal occlusion are the fibrin(ogen) receptors. These are $\alpha v \beta 3$, $\alpha m \beta 2$, $\alpha II \beta 3$ and $\alpha 5 \beta 1$ ^{57, 77-80}.

Due to the lack of integrin blocking reagents that could be used in animal studies of atherosclerosis and our previous results on the effectiveness of $\alpha 5 \beta 1$ and fibronectin antibodies to inhibit smooth muscle

cells-fibrin interactions, we were interested in identifying small molecular weight integrin antagonists that could specifically prevent human smooth muscle cell interactions with fibrin. We tested four different molecules, penRGD, G4120, VCAM345 and Kistrin^{94, 158-160}. They have all been shown to disrupt cell-matrix interactions by blocking specific integrins. PenRGD and G4120 are cyclic peptides that predominantly inhibit the $\alpha v\beta 3$ and $\alpha IIb\beta 3$ integrins. VCAM345 blocks both the $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins. Kistrin is a snake venom disintegrin that blocks the $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 5$ integrins. Of all of the antagonists, Kistrin was the most effective in preventing human smooth muscle cell interactions with fibrin. This suggests that a pan-integrin inhibitor may be the ideal molecule to test in animal models of both restenosis and atherosclerosis progression.

Materials and Methods

Reagents

The cyclic peptide, penGRGDSPCA, was purchased from Gibco/BRL (Gaithersburg, MD).⁹⁴ The cyclic peptide G4120 and the disintegrin, Kistrin, were gifts from Genentech, Inc. (South San Francisco, CA)^{158, 160}. VCAM345 was provided by Sherman Fong and Dave Jackson of Genentech, Inc. (South San Francisco, CA)²⁸. PenGRGDSPCA (penRGD) and G4120, are cyclic RGD peptides^{94, 158}. Both peptides have been shown to inhibit interactions of $\alpha v\beta 3$ with vitronectin and $\alpha IIb\beta 3$ with fibrinogen. While G4120 displays similar activities for both $\beta 3$ integrins, penRGD appears to be approximately 10-fold more potent for $\alpha v\beta 3$ over $\alpha IIb\beta 3$ ^{158, 161}. The third molecule, VCAM345, has been shown to be a potent inhibitor of $\alpha 4$ interaction with VCAM as well as $\alpha 5\beta 1$ interaction with fibronectin¹⁵⁹. Kistrin efficiently blocks $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 5$ interactions^{158, 160, 162} (S. Bodary, unpublished data).

Cell Culture

Human Newborn 18 smooth muscle cell strain, originally derived from the aorta of a newborn infant (2-day old) autopsy, has been described previously^{107 3}. This strain was immortalized by transfection with the type

16 human papilloma virus proteins E6 and E7, which interact with p53 and Rb¹⁰⁸. This strain is now referred to as Human Newborn 18 E6E7 (HNB18E6E7). We have previously shown that HNB18E6E7 smooth muscle cells interact with fibrin exactly as a non-immortalized smooth muscle cell strain derived from the aorta of a newborn¹²⁵. Because HNB18E6E7 smooth muscle cells grow better in culture, we have chosen to use this strain in these studies. HNB18E6E7 smooth muscle cells were grown in Waymouth medium (GIBCO/BRL; Gaithersburg, MD) containing 0.23% sodium bicarbonate, 0.35 mg/ml L-glutamine, 1 mmol/L sodium pyruvate, and 0.01 mmol/L non-essential amino acids (GIBCO/BRL; Gaithersburg, MD). This basic medium was supplemented with 10% FCS (HyClone Laboratories, Inc.; Logan, UT). HNB18E6E7 smooth muscle cells were harvested at passages 25-27.

Plasma, Gelatin Sepharose Treated Plasma, Recombinant Fibrinogen and Thrombin

Citrated human blood from healthy donors was centrifuged at 150 x g for 15 minutes at 20°C and the subsequent platelet-rich plasma was then centrifuged at 1400 x g for 20 minutes at 20°C. Part of the platelet poor plasma was stored at -70°C. The rest of the platelet-poor plasma was placed on a gelatin sepharose 4B column (Pharmacia; Uppsala, Sweden) that had previously been equilibrated with a buffer containing 0.05 mol/L Tris-HCL, pH 7.5, 5 mmol/L benzamidine and 0.02% sodium azide. The gelatin sepharose treated plasma was collected and the extent of fibronectin depletion was determined by Western Blot analysis^{109, 110}. In all cases, 80-90% of the fibronectin was removed from the plasma. The gelatin sepharose treated plasma was stored at -70°C. The production, purification and characterization of recombinant fibrinogen have already been described^{111, 112}. Human α -thrombin was purchased from Calbiochem (La Jolla, CA).

Adhesion Assay

This assay has already been described¹²⁵. Briefly, equal amounts of a fibrinogen solution (1:5 dilution of plasma) and a thrombin solution (5 units/ml) were added to each well of a 96 well immunosorbant plastic plate

(Nunc; Naperville, IL) and stored overnight at 4°C. The wells were washed twice with PBS and blocked with 10 mg/ml of BSA-PBS for 1 hour at 37°C. The smooth muscle cells (50,000 cells/well) were pre-incubated for 15 minutes at room temperature with the indicated peptides in serum-free Waymouth medium containing 1 mg/ml of BSA, and then allowed to adhere to the substrate at 37°C/5% CO₂ for 1 hour. Non-adhering cells were washed off using PBS that was pre-warmed at 37°C, and the adhering cells were fixed in 4% paraformaldehyde and stained with 4% paraformaldehyde containing 0.5% toluidine blue. The color was solubilized using 1% SDS and color intensity was measured using an ELISA reader set at 595nm¹¹³. All experiments were carried out independently at least twice with n=3 for each group.

Plasma Clot Contraction Assay

This assay is based on the one published by Ylanne *et al.* and has been modified as described previously^{67, 125}. Briefly, cultured human smooth muscle cells were harvested using trypsin-EDTA, washed twice in 0.5mg/ml of soybean trypsin inhibitor (Sigma Chemical Co.; St. Louis, MO), and resuspended at a concentration of 10⁷ cells/ml in serum-free, Hepes (25mmol/L) buffered-DMEM (GIBCO/BRL; Gaithersburg, MD). Fibrinogen solution (1:5 dilution of plasma) was added to the smooth muscle cells in a 6mm X 50mm pre-siliconized, autoclaved borosilicate test tube. Lastly, a mixture containing 28mmol/L CaCl₂ and 5 units/ml of thrombin in serum-free, Hepes-DMEM was added to the test tube. The test tubes were incubated at 37°C with 5% CO₂. Pictures were taken with an Olympus OM2 camera using Kodak Ektachrome T64 slide film (Rochester, NY) and a Zuiko 50mm f3.5 macro lens at t=0 and at time points specified in the figure legends. The slide was projected onto a piece of paper and the clot was traced. From the tracing, a two dimensional area was calculated to determine the percent of clot contraction using the following equation.

$$\% \text{ Clot Contraction (t= 10 hrs)} = 100 - \frac{[\text{area t=10 hrs} \times 100]}{\text{area at t=0}}$$

The mean and the standard error (SE) were calculated (Microsoft Excel; Redmond, WA). All experiments were carried out independently at least twice with $n=3$ for each group. One representative experiment is shown.

Transwell Migration Assay

Migration assays were performed using Transwell tissue culture inserts with 8 μm pores (Costar, Cambridge, MA). The bottoms of the filters were coated with plasma (1:5 dilution) and thrombin (5 units/ml) and incubated coated side up at 37°C for 1 hour. Both sides of the filter were blocked for 1 hour at 37°C with a 2% BSA-PBS solution. Meanwhile, human smooth muscle cells were harvested in the same manner as in the clot contraction assay and preincubated with the stated concentration of peptides for 30 minutes at room temperature in a volume of 300 μl of Waymouth medium containing 1 mg/ml of BSA. 100 μl of cells were added to the top wells and 200 μl of Waymouth medium containing 1 mg/ml of BSA and 1 ng/ml of PDGF-BB were placed in the bottom well. The wells were incubated at 37°C for 6 hours. Cells remaining in the top wells were removed using a cotton swab. The filters were dipped in PBS, fixed in 4% paraformaldehyde and stained with 0.5% toluidine blue in 4% paraformaldehyde. Filters were washed in water to remove excess dye, cut from the wells and placed on glass slides. Cells were counted using a light microscope (magnification 20X) fitted with a grid-containing eye-piece. All experiments were carried out independently at least twice with $n=3$.

Recombinant Fibrin Clot Contraction Assay, Adhesion Assay and Migration Assay

The assays were similar to those described above except that recombinant fibrinogen^{111, 112} at a final concentration of 0.2 mg/ml, was used instead of whole plasma or gelatin sepharose treated plasma.

Statistical Analysis

StatView 4.5 for MacIntosh (Abacus Concepts, Inc., Berkeley, CA) was used to calculate the p-values for all of the data. Briefly, the percent clot contraction (contraction assay), the absorbance at 595nm (adhesion assay) or cell number (migration assay) was entered into the program. The mean,

variance, standard deviation and standard error were calculated. If the variance between groups differed more than 20 fold, transformation analysis was performed on the raw data. In this paper, the function used for the contraction and migration assays was the log (x) where x represents the % clot contraction or number of cells migrated to the bottom of the filter. For the adhesion assay, the function 1/x was used to transform the data, where x represents absorbance at 595nm. Using either the transformed data or raw data, an F-test was performed as well as an ANOVA. If the ANOVA conveyed a p-value less than 0.05, than a Fisher's PLSD was calculated. A p-value less than 0.05 indicates statistical significance.

Results

Adhesion of human smooth muscle cells to fibrin is inhibited by integrin peptide antagonists

We first tested the ability of the four integrin antagonists to inhibit smooth muscle cell adhesion to recombinant fibrin clots. Human smooth muscle cells preincubated with 200 μ M of the cyclic RGD peptide G4120 did not adhere well to recombinant fibrin clots as compared with the untreated control (p-value <0.001) (Figure 4.1A). VCAM345 also inhibited human smooth muscle cell adhesion to recombinant fibrin clots, but to a lesser extent at the same concentration (p-value <0.005) (Figure 4.1A). PenRGD, another cyclic RGD peptide, was a more potent inhibitor than G4120 at 200 μ mol/L (p-value<0.001). Neither of these peptides are considered to be potent inhibitors of α 5 β 1 interactions with fibronectin. The effect of penRGD was almost equal to Kistrin, a disintegrin. Kistrin had the most pronounced inhibitory effect on smooth muscle cell adhesion to recombinant fibrin clots. At 25 μ mol/L of Kistrin, cell adhesion was inhibited almost 90% (p-value<0.001) (Figure 4.1A).

We then looked at the effect of these peptides on cell adhesion to gelatin sepharose treated plasma clots, which have been depleted of fibronectin, a ligand for many integrins¹¹⁰. We have previously shown that cell adhesion to this matrix was largely through the plasma protein

vitronectin, an RGD containing matrix molecule¹²⁵. The data presented here also support this conclusion. The cyclic RGD peptides, G4120 at 200 $\mu\text{mol/L}$ and penRGD at 200 $\mu\text{mol/L}$, inhibited smooth muscle cell adhesion to gelatin sepharose treated plasma clots more than to recombinant fibrin (p -value <0.001) (Figure 4.1A and 4.1B). Kistrin at 25 $\mu\text{mol/L}$ blocked smooth muscle cell adhesion to gelatin sepharose treated plasma clots equally as well (p -value <0.001) (Figure 4.1B). VCAM345, an $\alpha 5$ and $\alpha 4$ integrin specific cyclic-peptide antagonist, had a modest effect (Figure 4.1B).

Finally we examined the effect of the peptides on smooth muscle cell adhesion to clots made from whole plasma, the matrix most likely to resemble a fibrin clot *in vivo*. smooth muscle cell adhesion to whole plasma clots was not significantly diminished by either the cyclic peptides, penRGD and G4120, or VCAM345 (Figure 4.1C). The failure of these peptides to inhibit adhesion on this substrate may be due to the numerous plasma proteins that are present in the clot that could be used by the cells for adhesion. Alone, none of these peptides were sufficient to block these interactions. However, the disintegrin Kistrin, which effectively blocks $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 5$ integrins, dramatically inhibited smooth muscle cell adhesion to whole plasma clots (p -value <0.001) (Figure 4.1C).

To explore the potency of Kistrin, we used a range of concentrations of Kistrin to inhibit smooth muscle cell adhesion to recombinant fibrin clots, gelatin sepharose treated plasma clots and whole plasma clots. We saw that a concentration as low as 0.1 $\mu\text{mol/L}$ of Kistrin was sufficient to block smooth muscle cell adhesion to recombinant fibrin and to gelatin sepharose treated plasma clots (Figure 4.2A and 4.2B). This concentration is approximately 10-fold above the IC_{50} for inhibition of adhesion of $\alpha\text{v}\beta 3$ expressing cells to vitronectin or fibrinogen¹⁶¹ and is in agreement with Kistrin being a inhibitor of both $\beta 1$ and $\beta 3$ integrins at nmol/L concentrations. To significantly block cell adhesion to whole plasma clots, a concentration of 25 $\mu\text{mol/L}$ was needed (Figures 4.1C and 4.2C).

We also looked at the ability of these antagonists to block smooth muscle cell adhesion to fibronectin and to vitronectin. Smooth muscle adhesion to fibronectin was significantly blocked by VCAM345 and by Kistrin (p -values <0.001)(Figure 4.3A). However, penRGD and G4120 also significantly inhibited cell adhesion to fibronectin (p -value <0.001 and p -

value=0.015, respectively). Cell adhesion to vitronectin was inhibited to a greater degree by both the cyclic RGD peptides as well as Kistrin (p-values<0.001). VCAM 345 also inhibited cell adhesion to vitronectin (p-value=0.039) (Figure 4.3B). Since $\alpha v\beta 3$ is a receptor for both fibronectin and vitronectin, the data with G4120 and penRGD inhibition (Fig 4.3A) are not surprising. In fact, both $\alpha 5\beta 1$ and $\alpha v\beta 3$ probably mediate adhesion to fibronectin in smooth muscle cells. These data agree with our observation that G4120 is a much better inhibitor of $\alpha v\beta 3$ than of $\alpha 5\beta 1$. The IC₅₀'s in a protein-protein assay are 8 nmol/L for $\alpha v\beta 3$ /vitronectin and 2340 nmol/L for $\alpha 5\beta 1$ /fibronectin (S. Bodary, unpublished data)¹⁶¹. Based on the data in Figures 1A and 3A, penRGD is a better fibronectin receptor antagonist than G4120. Thus VCAM345, G4120 and penRGD are able to inhibit to various degrees cell adhesion to fibronectin and vitronectin, but only Kistrin is able to inhibit cell adhesion profoundly to both matrices.

Smooth muscle cell contraction of fibrin clots and migration to fibrin is only inhibited by Kistrin

Smooth muscle cell adhesion to fibrin is not the only event that could occur *in vivo*. After the initial adhesion to the matrix, smooth muscle cells can interact with fibrin in a three dimensional manner and this interaction could affect vessel narrowing during atherosclerosis progression. To determine whether our Integrin antagonists can inhibit smooth muscle cell interaction with a fibrin clot in a three dimensional system, we tested whether smooth muscle cells that are immersed in a clot can contract the clot in the presence of the inhibitors. Contraction of recombinant fibrin clots, gelatin sepharose treated plasma clots or whole plasma clots by smooth muscle cells was not significantly inhibited by penRGD, G4120 or VCAM345 (Figures 4A, 4B and 4C). Only Kistrin was able to inhibit smooth muscle cell contraction of fibrin clots and in all cases this inhibition was greater than 80% (p-values<0.001).

A second event that could occur once smooth muscle cells have adhered to the fibrin matrix is the migration of smooth muscle cells on and through a fibrin clot. To test whether these antagonists can effect cell migration on fibrin, we looked at how smooth muscle cells are able to migrate to a fibrin clot in the presence of these inhibitors using a transwell

migration assay system. When we coated filters with either recombinant fibrin, gelatin sepharose treated plasma clots or whole plasma clots, we found that only Kistrin was able to significantly block smooth muscle migration to these sources of fibrin (figure 5A, 5B, 5C). penRGD, G4120 and VCAM345 had some inhibitory effect, but Kistrin consistently inhibited migration when using all three sources of fibrinogen. These data along with our previous data suggest that human smooth muscle cell contraction of fibrin clots and migration on fibrin use multiple Integrins including $\alpha 5\beta 1$ and $\alpha v\beta 3$.

Discussion

Numerous attempts have been made to develop therapeutic reagents that prevent restenotic narrowing. Recently, these attempts have included peptide antagonists to the Integrin family of matrix receptors and such peptides have been tested in animal models of vascular diseases¹⁵⁰⁻¹⁵². These studies demonstrated that the peptides were effective in preventing intima thickening; however, as we have already noted, a more relevant problem may be remodeling³⁷. Indeed to our knowledge, the only published data showing inhibition of remodeling after vascular injury is ours³⁷. By blocking fibrin formation, we were able to inhibit lumen narrowing. Because fibrin is a matrix molecule, its ability to interact with cells depends on cell surface matrix receptors such as integrins. However, in the studies using integrin antagonist peptides the cell-matrix interactions and the integrins being affected by the peptides were not identified. The investigators assumed that the cyclic RGD peptide, an αv integrin antagonist⁹⁴, was preventing smooth muscle proliferation and migration.

Ikari *et al.* recently demonstrated the presence of fibrin(ogen) in human carotid lesions and the predominance of $\beta 1$ integrins over $\beta 3$ integrins on smooth muscle cells co-localized with fibrin(ogen)⁶⁶. Smooth muscle cell interaction with intramural fibrin could play a critical role in remodeling. In this study we have attempted to identify integrin antagonists that inhibit human smooth muscle cell interaction with fibrin.

We have used four different integrin antagonists in three different assays (adhesion assay, clot contraction assay and migration assay) using three different sources of fibrinogen (recombinant, gelatin sepharose treated and whole plasma) and characterized their effects on smooth muscle cell interaction with fibrin.

Two of these molecules are cyclic RGD peptides (penRGD and G4120) that have been used in numerous animal studies^{150-152, 163}. We observed that they were effective inhibitors of smooth muscle cell adhesion to recombinant fibrin clots and gelatin sepharose treated plasma clots, but not to whole plasma clots. Because we have shown previously that adhesion to gelatin sepharose treated plasma clots is via the vitronectin protein, the effectiveness of the cyclic RGD peptides in inhibiting smooth muscle adhesion to this matrix was not surprising¹²⁵. However, neither peptide was able to inhibit contraction of fibrin clots or migration of smooth muscle cells on fibrin clots. This could be related to the dose used in this study. Previously, we showed that penRGD was able to inhibit contraction of gelatin sepharose treated plasma clots at a much higher concentration (500 μM)¹²⁵. The ineffectiveness of these peptides to block smooth muscle cell contraction and migration on fibrin clots could be due to the inability of the peptides to inhibit all the fibronectin and vitronectin receptors on the cell surface.

Based on our previous results which demonstrated that antibodies against the $\alpha 5\beta 1$ integrin and fibronectin are able to inhibit human smooth muscle cell interaction with fibrin, we tested a cyclic peptide, VCAM345, that has been shown to inhibit the integrin subunit $\alpha 5$ interaction with fibronectin and $\alpha 4$ interaction with VCAM in an ELISA assay (M. Beresini, unpublished data)¹⁵⁹. We found that VCAM345 inhibited smooth muscle adhesion to recombinant fibrin clots, but not to gelatin sepharose treated plasma clots or whole plasma clots. It is likely that human smooth muscle cell adhesion to these matrices occurs via vitronectin binding to $\alpha v\beta 3$ which is not inhibited by VCAM345. However, VCAM345 also did not inhibit contraction of fibrin clots or migration of smooth muscle cells on fibrin clots. Perhaps in these cellular events, both the $\alpha v\beta 3$ and the $\alpha 5\beta 1$ integrins need to be blocked.

Kistrin, a disintegrin derived from the venom of the viper snake, *Agkistrodon rhodostoma*, has been shown in protein based assays to inhibit vitronectin interactions with αv integrins, fibrinogen interactions with $\alpha IIb\beta 3$ and $\alpha v\beta 3$ and fibronectin interaction with $\alpha 5\beta 1$. In addition, Kistrin is a less potent though effective inhibitor of $\alpha 4\beta 1$ interaction with VCAM and $\alpha L\beta 2$ and $\alpha m\beta 2$ interactions with ICAM-1 (S. Bodary and S. Fong, unpublished data)^{160, 164, 165}. In our system, Kistrin was the most effective inhibitor of smooth muscle cell adhesion, contraction and migration to all three sources of fibrin (recombinant fibrin clots, gelatin sepharose treated plasma clots and whole plasma clots).

The data presented here suggest that adhesion to fibrin clots is mediated by both the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins. The ability of different peptides to inhibit this interaction depends on the integrin specificity of the peptide. PenRGD was considered an αv antagonists because it was effective in inhibiting adhesion of rat kidney cells to vitronectin and not to fibronectin at 100 μM . However, at higher concentrations of penRGD, cell adhesion to fibronectin was slightly inhibited⁹⁴. In our hands, penRGD at 100 μM is able to inhibit smooth muscle cell interaction with fibronectin and vitronectin. This difference in results could be due to the different species and cell types used in each experiment. Others have shown that cyclic RGD peptides are able to inhibit other non- αv integrins such as $\alpha 4\beta 1$ interaction with VCAM¹⁶⁶. In this case the authors did not believe that the RGD motif contributed to the inhibitory effect, but that the tertiary structure of the cyclic peptide was responsible for the activity. This could explain why a cyclic RGD peptide was effective in preventing hyperplasia in several animal models of atherosclerosis^{150, 152}.

VCAM345 was effective only in inhibiting smooth muscle cell adhesion to recombinant fibrin. Unlike the anti- $\alpha 5\beta 1$ antibodies, VCAM345 did not affect contraction of fibrin clots. This could be due to several factors. One, the antibodies may have a higher affinity for the integrin and thus be more effective. Two, because VCAM345 blocks both $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins, it may not be as specific for $\alpha 5\beta 1$ as the anti- $\alpha 5\beta 1$ antibody. Three, the antibodies could be having another effect on the cells besides inhibiting cell-matrix interactions. There have been examples of cross-talk between integrins in other cell types where ligation of one integrin affects the activity

of another¹⁶⁷⁻¹⁶⁹. Therefore, the anti- $\alpha 5\beta 1$ antibodies may affect the $\alpha v\beta 3$ integrin binding affinity.

Of all of the molecules, only Kistrin was effective in preventing smooth muscle cell interaction with all three sources of fibrin in all three assays. The ability of Kistrin to inhibit both $\beta 1$ and $\beta 3$ integrins may be due to the RGD loop in the protein. Based on 2D NMR studies, the RGD loop is thought to be present in numerous different conformations which may explain why Kistrin is such a good inhibitor of many RGD sensitive integrins, such as $\alpha v\beta 3$, $\alpha IIb\beta 3$, $\alpha 5\beta 1$ and the $\beta 2$ integrins, $\alpha m\beta 2$ and $\alpha L\beta 2$ ¹⁷⁰. Other members of the disintegrin/snake venom family, such as Batroxostatin, have also been shown to block multiple integrins¹⁷¹. The fact that a non-selective integrin antagonist was the most effective in preventing smooth muscle cell interaction with fibrin was not surprising. When we combined antibodies against the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins, we observed a greater inhibition of smooth muscle cell interaction with fibrin than with the individual antibodies alone (K. Yee, unpublished data). The data presented in this manuscript suggest that future successful therapies for vascular diseases may include drugs that inhibit the various fibrinogen receptors such as $\alpha v\beta 3$, $\alpha IIb\beta 3$, $\alpha 5\beta 1$ and $\alpha m\beta 2$.

The potency of Kistrin leads us to believe that the most effective integrin antagonists may be small molecules that are less specific than those currently in use. Studies using Kistrin in animal models have been carried out or are in progress. One group tested the anti-thrombolytic effect of Kistrin in conjunction with recombinant tissue plasminogen activator in a canine model of coronary artery thrombosis¹⁷². The investigators found a decrease in reperfusion time, increase in bleeding time that became normal at the end of the experiment, decrease in platelet aggregation, and a higher degree of patency. This study suggest that a Kistrin-like antagonist could be a promising pharmaceutical agent for prevention of vascular diseases.

Figure 4.1: Inhibition of cell adhesion to fibrin clots using anti-integrin peptides

(A) Integrin antagonists VCAM345, penRGD, G4120 and Kistrin. Recombinant fibrin and thrombin were coated onto a 96 well plate overnight at 4°C. Human smooth cells were preincubated for 15 minutes at room temperature with either 25 $\mu\text{mol/L}$ of Kistrin, 200 $\mu\text{mol/L}$ VCAM345, 200 $\mu\text{mol/L}$ G4120 or 200 $\mu\text{mol/L}$ penRGD peptides. The cells were allowed to adhere to the substrate for 1 hour at 37°C, and non-adhering cells were washed off with PBS. The adhering cells were fixed with 4% paraformaldehyde and stained with toluidine blue. The color intensity was measured by an ELISA reader at 595nm. Values represent the mean of the treated divided by the mean of the control \pm standard error (n=3). (B) The procedure is the same as in 1A except that gelatin sepharose treated plasma clots was used as a substrate. (C) The procedure is the same as in 1A except that plasma clots was used as a substrate.

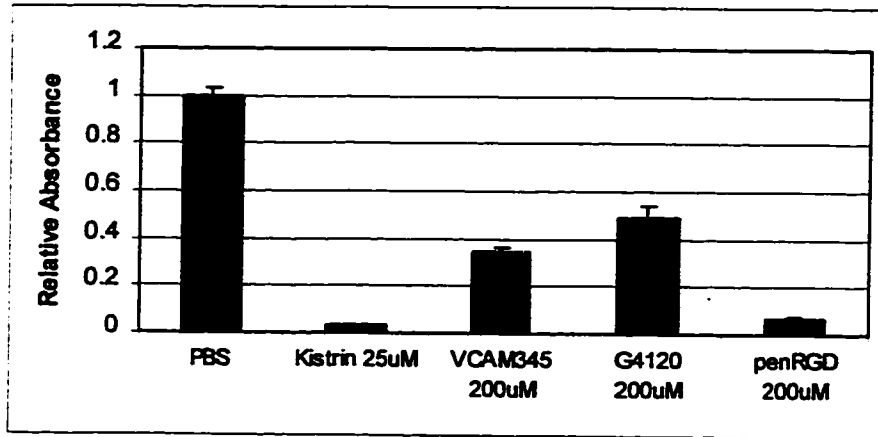
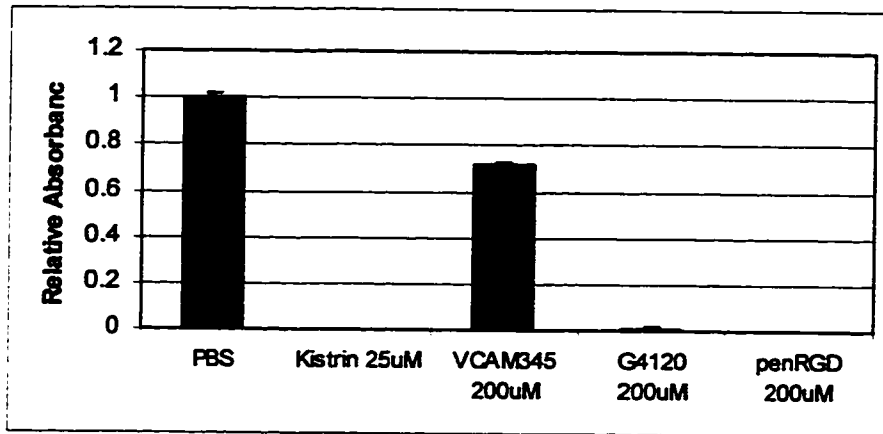
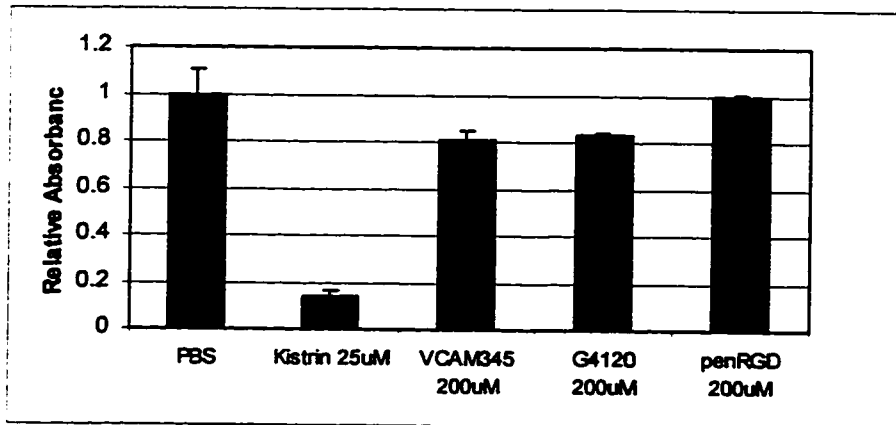
A**B****C**

Figure 4.2: Kistrin inhibits cell adhesion to fibrin clots using

(A) Recombinant fibrin and thrombin were coated onto a 96 well plate overnight at 4°C. Human smooth cells were preincubated for 15 minutes at room temperature with either 0.1 µmol/L, 0.25 µmol/L, 0.5 µmol/L, 0.75 µmol/L or 1 µmol/L of Kistrin. The cells were allowed to adhere to the substrate for 1 hour at 37°C, and non-adhering cells were washed off with PBS. The adhering cells were fixed with 4% paraformaldehyde and stained with toluidine blue. The color intensity was measured by an ELISA reader at 595nm. Values represent the mean of the treated divided by the mean of the control ± standard error (n=3). **(B)** The procedure is the same as in 2A except that gelatin sepharose treated plasma clots was used as a substrate. **(C)** The procedure is the same as in 2A except that plasma clots was used as a substrate.

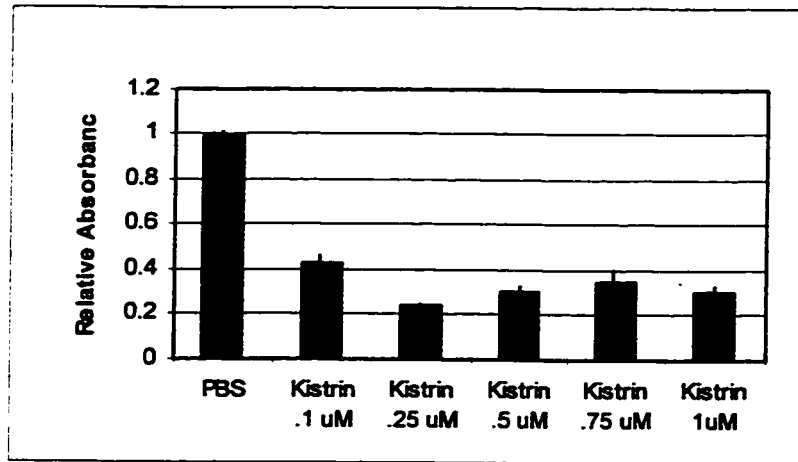
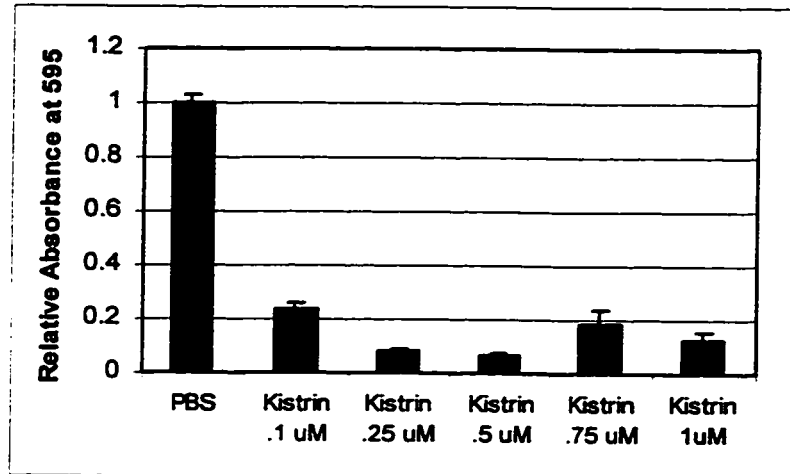
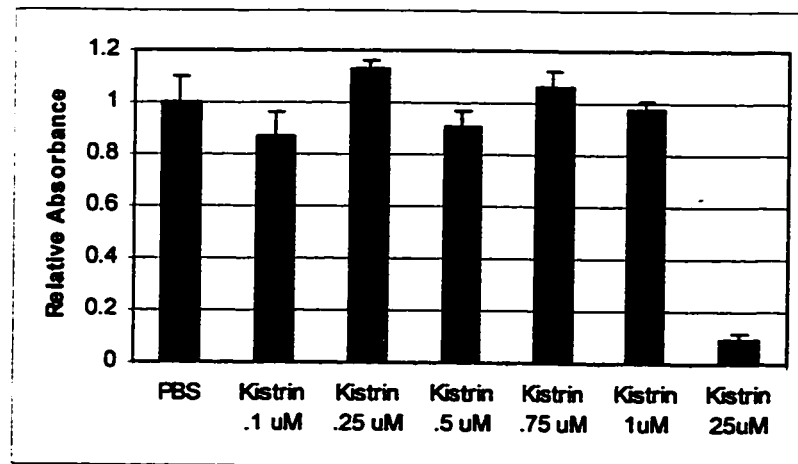
A**B****C**

Figure 4.3: Inhibiting cell adhesion to fibronectin and vitronectin

(A) Human smooth muscle cells were untreated or treated with either 50 $\mu\text{mol/L}$ of Kistrin, 200 $\mu\text{mol/L}$ of VCAM345, 200 $\mu\text{mol/L}$ G4120 or 200 $\mu\text{mol/L}$ penRGD peptides for 30 minutes at room temperature. Recombinant fibrinogen and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 8 hours. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3). **(B)** The procedure is the same as in 4A except that gelatin sepharose treated plasma was used in the assay. **(C)** The procedure is the same as in 4A except that whole plasma was used in the assay.

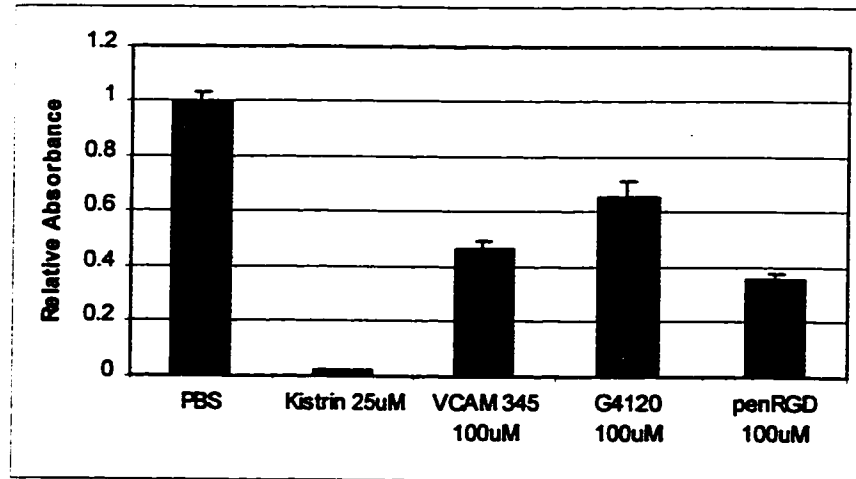
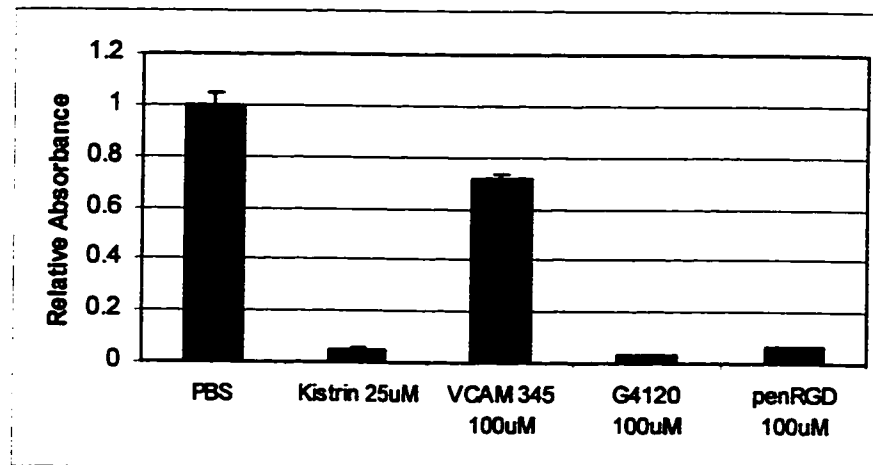
A**B**

Figure 4.4: Effect of integrin antagonists on fibrin clot contraction by HNB18E6E7

(A) Human smooth muscle cells were untreated or treated with either 50 $\mu\text{mol/L}$ of Kistrin, 200 $\mu\text{mol/L}$ of VCAM345, 200 $\mu\text{mol/L}$ G4120 or 200 $\mu\text{mol/L}$ penRGD peptides for 30 minutes at room temperature. Recombinant fibrinogen and human thrombin were added to the cells to form a clot, and the clot was incubated at 37°C for 8 hours. The % clot contraction was calculated as described in methods. Values represent the mean \pm standard error (n=3). (B) The procedure is the same as in 4A except that gelatin sepharose treated plasma was used in the assay. (C) The procedure is the same as in 4A except that whole plasma was used in the assay.

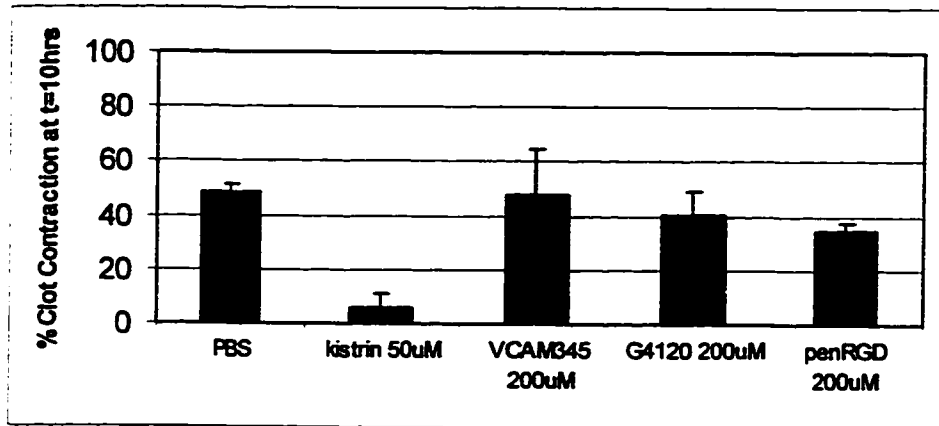
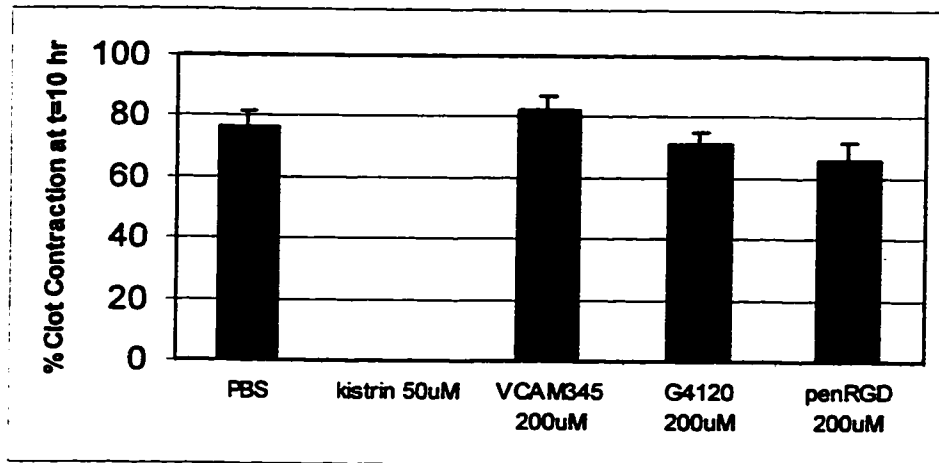
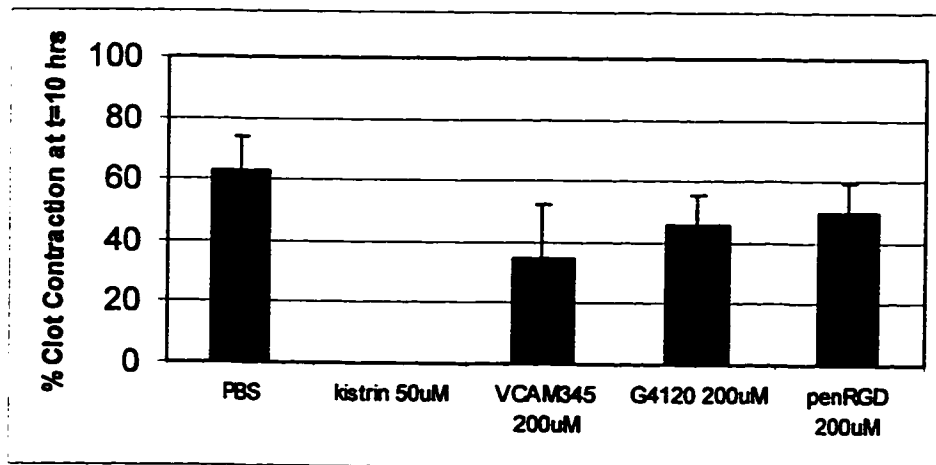
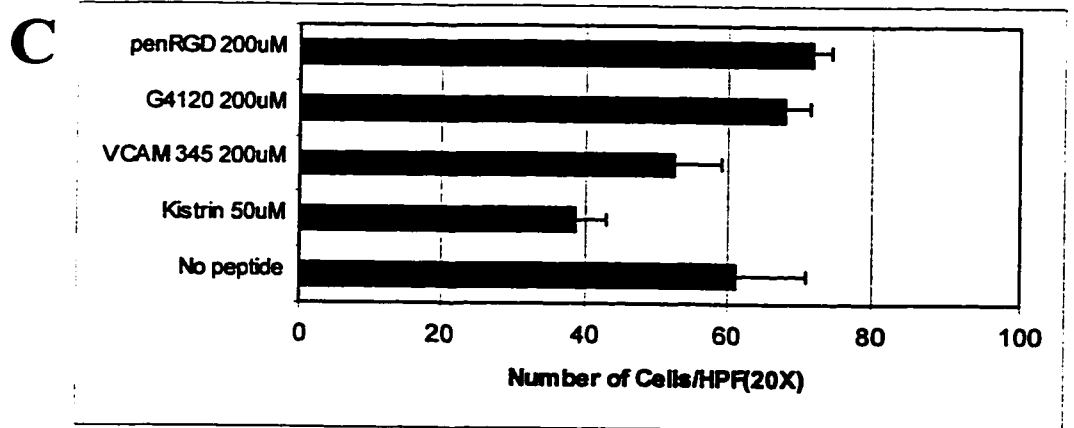
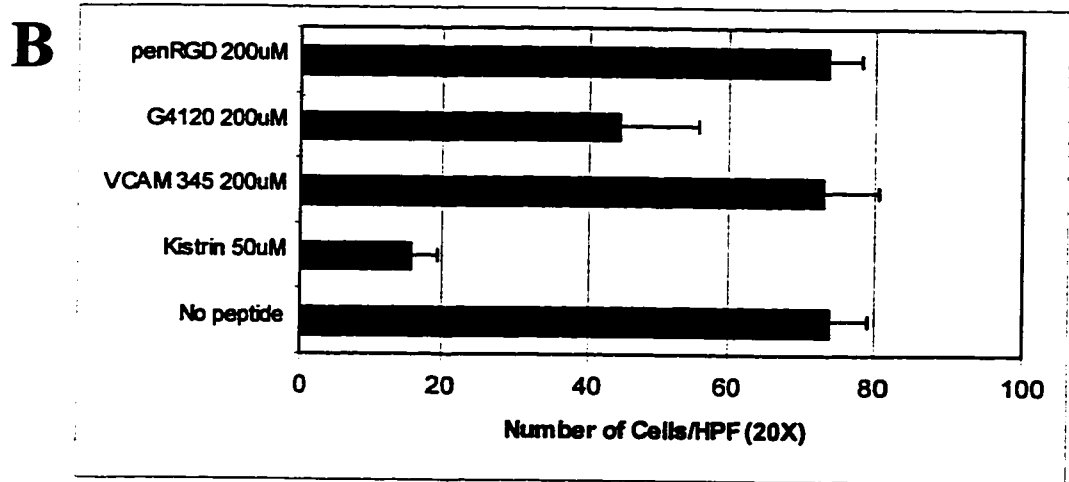
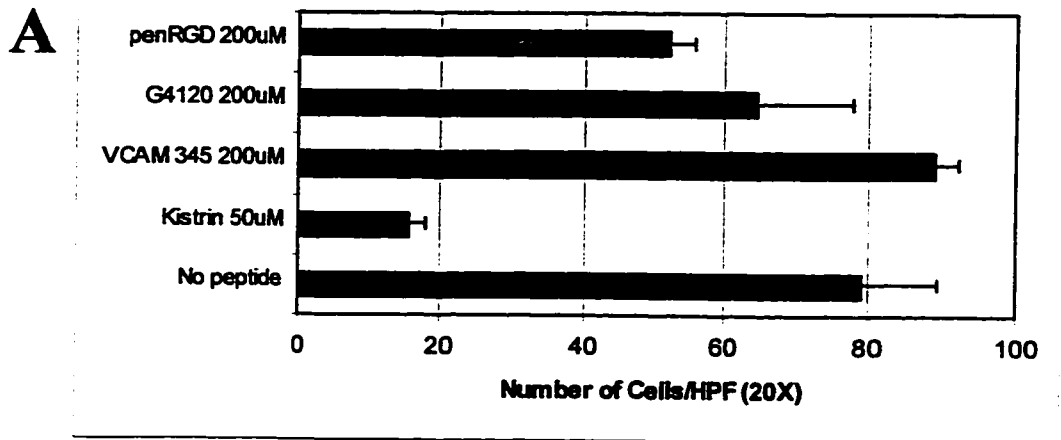
A**B****C**

Figure 4.5: Inhibiting cell migration on fibrin clots

(A) Human smooth muscle cells were untreated or treated with either 50 $\mu\text{mol/L}$ of Kistrin, 200 $\mu\text{mol/L}$ of VCAM345, 200 $\mu\text{mol/L}$ of G4120 or 200 $\mu\text{mol/L}$ of penRGD peptides for 30 minutes at room temperature. The cells were added to the upper chamber of a Transwell apparatus that had previously been coated with recombinant fibrin. The cells were induced to migrate for 6 hours at 37°C using PDGF-BB. At the end of the assay, non-migrating cells were removed from the upper chamber and the migrating cells were fixed with 4% paraformaldehyde, stained with toluidine blue and counted under a light microscope. Values represent the mean number of cells \pm standard error (n=3). (B) The procedure is the same as in 5A except that gelatin sepharose treated plasma was used to coat the bottoms of the filters. (C) The procedure is the same as in 5A except that whole plasma was used to coat the bottoms of the filters.



Chapter 5: Fibrin and Integrin Expression in Human Carotid Arteries: Role of $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins in Smooth Muscle Cells Spreading and Migration

This work was submitted to *Circulation* by Yuji Ikari, Karen O Yee, Thomas S. Hatsukami and Stephen M. Schwartz.

Introduction to Chapter 5

Interest in the role of $\beta 3$ integrins in the vascular response to injury has been heightened by clinical use of an anti-integrin antibody, ReoPro, which blocks $\alpha v\beta 3$ and $\alpha IIb\beta 3$ integrins¹⁷³. ReoPro successfully reduced late clinical events as well as acute complications following angioplasty in patients with unstable angina^{114, 148, 174-178}. The acute effects were to be expected since $\alpha IIb\beta 3$ expressed on platelets is a major receptor for fibrin and ReoPro prevents platelet aggregation¹⁷⁹. Anti-platelet effects, however, might be expected to be important only in the early phases of the vessel's response to angioplasty. The other target of ReoPro, $\alpha v\beta 3$, has also been proposed to play a role in preventing restenosis. $\alpha v\beta 3$ is expressed on smooth muscle cells⁶⁵, and is also a receptor for fibrin as well as other matrices¹⁸⁰. Animal studies using peptide antagonists to $\alpha v\beta 3$ suggest that such drugs may be effective in blocking smooth muscle cell migration^{151, 152}. This has led to the hypothesis that the long term effects of ReoPro depend is due to its ability to inhibit smooth muscle cell migration by blocking cell-matrix interactions via the $\alpha v\beta 3$ integrin.

We tested this hypothesis by studying the integrin expression pattern on intimal smooth muscle cells located in vessels that have undergone spontaneous injury as well as by analyzing the integrins involved in cell spreading and migration assay on fibrin *in vitro*. We focused on the interaction of smooth muscle cells with fibrin for several reasons. First, fibrin is the major provisional matrix formed at the site of injury following angioplasty¹⁸¹. Second, an elevated level of plasma fibrinogen is a risk factor for restenosis¹⁸². Third, because the success of ReoPro may not be primarily due to the blocking of the fibrin receptor on platelets, other $\beta 3$ integrins on other cells types should be explored as potential targets for Reopro. One example is the fibrin receptor on smooth muscle cells. Fourth, we have recently shown that vascular narrowing, though not neo-intimal formation, is blocked when animals undergo sequential balloon injury in the presence of a Factor VIIa inhibitor³⁷. This suggest that fibrin may be important in narrowing. Finally, while collagen substrates are also of interest, *in vitro* studies are difficult to carry out because the expression of

one of the main receptors for collagen, integrin $\alpha 2\beta 1$, is a culture artifact for human smooth muscle cells. $\alpha 2\beta 1$ is not seen in these cells *in vivo*⁶⁴.

Materials and Methods

Antibodies and peptides

MAB LM609 (anti-human integrin $\alpha v\beta 3$) and MAB JSB5 (anti-human integrin $\alpha 5\beta 1$) were purchased from Chemicon International (Temecula, CA). MAB P4C10 (anti human integrin $\beta 1$) was purchased from GIBCO/BRL (Gaithersburg, MD). MAB Y2/51 (anti-human $\beta 3$ integrin) and MAB anti α -actin were purchased from Boehringer Mannheim (Germany). MAB anti-CD68 was purchased from DAKO (Denmark). MAB anti-human tissue factor type 1 was purchased from Calbiochem (La Jolla, CA). MAB anti-fibrinogen and fibrin βB chain (#350) was purchased from American Diagnostica (Greenwich, CT) and MAB T2G1 (anti-fibrin β chain) was from Centocor (Malvern, PA). GRGDSP, GRGESP, GRGDNP and GPenGRGDSPCA were purchased from GIBCO/BRL(Gaithersburg, MD).

Cell spreading assay in plasma clots

Human Newborn 18 smooth muscle strain (HNB18E6E7) has been described previously and was originally derived from the aorta of a newborn infant (2-day old) autopsy^{107, 108}. The integrin expression pattern of this cell line has been reported previously¹²⁵. The expression of $\alpha v\beta 3$ is low in this cell line. HNB18E6E7 cells were detached by a brief exposure to 0.05% trypsin - 0.02% EDTA. Trypsin was inactivated by excess amounts of soy bean trypsin inhibitor. After washing in PBS, cells were resuspended in serum-free DMEM containing 25 mM HEPES (1.3×10^5 / 105 μ l). Antibodies were added to the cell suspension and incubated for 30 min. Sodium-citrate human plasma from a normal healthy subject with was diluted to 20% by DMEM containing 25 mM HEPES. Cells were mixed well with 60 μ l of 20% plasma in 8-well slide chambers. Subsequently, 60 μ l of thrombin (5 units/ml) in DMEM containing 25 mM HEPES and 28 mM CaCl_2 was added. Clots were formed in a few minutes after addition of thrombin. The slide

chamber was incubated at 37°C with 5% CO₂ for 24 hours. The number of spread cells and the number of rounded cells were counted in 3 separate fields for each well using a microscope. Mean value of the number of spread cells was calculated.

Migration assay into fibrin clots

We modified the fibrin invasion/migration assay originally reported by Naito *et al*¹⁸³, because in the original method the use of antibodies to inhibit migration was not possible. Fibrinogen was diluted to 4.1 mg/ml (final concentration 3.0 mg/ml) with serum-free DMEM containing 25 mM HEPES. Thrombin (5 units/ml) was added to DMEM containing 25 mM HEPES and 28 mM CaCl₂. 733 µl of fibrinogen solution and 266 µl of thrombin solution were added to each well of a 6-well plate. The plate was incubated at 37°C with 5% CO₂ for 1h. HNB18E6E7 cells were detached by a brief exposure to 0.05% trypsin - 0.02% EDTA. Trypsin was inactivated by excess amounts of soy bean trypsin inhibitor. After washing in PBS, cells were resuspended in serum-free DMEM containing 25 mM HEPES (1.5 X 10⁶ / 400 µl). Antibodies were added to the cell suspension at the total concentration of 20 µg/ml and incubated for 30 min. Cells were placed on the fibrin and incubated for 3 h at 37°C. After most of the cells reached the bottom of the fibrin coated well, media was gently removed. 733 µl of fibrin solution containing fetal bovine serum (final 5%) and 266 µl of thrombin solution were added to the cells to create a fibrin sandwich with cells between the two layers. Cell migration into the overlaid fibrin was observed at 24 h. Numbers of migrated cells were counted using a phase-contrast microscope. Migrated cells were easily detected by focusing into the top clot. In this assay system, migration into the bottom fibrin layer was never observed.

Carotid arteries

Eighty four carotid artery segments were obtained from 14 patients (all male, 58 to 81 years old) undergoing carotid endarterectomy. The endarterectomy samples were dissected in saline at 4°C and the specimens were frozen in OCT compound (Miles) and stored at -70°C.

Western Blot analysis

This method has been described previously⁶⁵. In brief, cell lysates were made from fresh carotid endarterectomy samples, from a fresh normal carotid artery obtained from an autopsy that died of liver disease and from a fresh thrombi in an abdominal aortic aneurysm. Tissues were homogenized in extraction buffer (PBS with 1% Triton X-100 and protease inhibitors) and allowed to incubate on ice for 20 min. The cell lysates were centrifuged twice at 16,000 x g for 10 min and the supernatants were kept as samples. Protein amount was measured using the Bio-Rad protein assay kit (Hercules, CA) and 10 μ g of protein was loaded in each lane. After samples were separated by electrophoresis on 8% polyacrylamide-SDS gels under reducing conditions, the proteins were transferred to polyvinylidene difluoride membranes (DuPont NEN). The membranes were blocked with 5% non-fat dry milk in buffer (10 mmol/l Tris base, pH 8, 150 mmol/l NaCl, and 0.05% Tween 20) at room temperature for 1 hour. After washing, the blots were incubated for an additional hour with a 1:150 dilution of MAb #350 or a 1:150 dilution of MAb T2G1 in buffer containing 5% non-fat dry milk. This was followed by a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson Immunolabs). The proteins were visualized by the addition of a chemiluminescence reagent to the membrane according to the manufacturer's instructions (DuPont NEN).

It is difficult to distinguish fibrin β chain from fibrinogen β B chain by molecular weight because the difference between the two is less than 2% (14 amino acids). In order to distinguish fibrin from fibrinogen, we compared binding of two antibodies, MAb #350 and MAb T2G1. MAb #350 reacts with fibrin β chain, fibrinogen β B chain and degradation product X and Y but does not react with degradation product D or E. MAb T2G1 reacts with fibrin β chain (β 15-42) and does not react with fibrinogen since the epitope appears after thrombin cleavage of fibrinopeptide B from the β chain. Thus, a sample stained positive with both antibodies was considered to be fibrin and a sample stained positive with #350 but negative with T2G1 was considered to be fibrinogen¹⁸⁴. The specificity of T2G1 has already been shown¹⁸⁵⁻¹⁹¹. But we performed both immunohistochemistry and western blot analysis to confirm the specificity in our system. T2G1 is specific for fibrin in our control samples (data not shown).

Immunohistochemistry

Immunohistochemistry was performed as described previously⁶⁵. In brief, the cryostat sections were fixed in 10% neutral-buffered formalin for 5 min at room temperature and internal peroxidase was blocked by incubating the sections in 3% hydrogen peroxide for 5 min at room temperature. Blocking was performed with 10% normal horse serum in PBS containing 1% BSA. Slides were incubated with primary antibody for 1h at room temperature: the titer was 1:500 for LM609 and JSB5, 1:200 for Y2/51, 1:150 for T2G1 and #350, 1:25 for anti-tissue factor antibody, 1:250 for anti- α -actin, 1:1000 for P4C10 and 1:8000 for CD68. A biotinylated horse anti-mouse secondary antibody was then applied for 30 min, followed by an avidin-biotin-peroxidase conjugate (ABC Elite, Vector Labs) for 30 min at room temperature. Then 3,3'-diaminobenzidine with nickel chloride was added to yield a black reaction product, and methyl green was used as a nuclear counterstain.

Fibrin positive area

Fibrin positive area stained with MAb T2G1 was quantified on cross sectional slides. We calculated the positive circumference / total circumference ratio. Twenty slides of diffuse intimal thickening and thirty-four slides of advanced lesion were addressed. The number of slides were low because only the tissues that were cut cross-sectionally were used. As a control for MAb T2G1, seven slides of coronary arterial diffuse intimal thickening from heart transplant recipients were stained.

Determining positive integrin expression on smooth muscle cells co-localized with fibrin

First, all areas where smooth muscle cells co-localized with fibrin were noted. These areas were identified by H&E staining and included diffuse intimal thickening, fibrous cap and regions of intramural hemorrhage. Intramural hemorrhage was confirmed by the presence of sideroblasts which are hemosiderin rich macrophages. Some smooth muscle cells were co-localized with fibrin that was found deep in the intima and below the necrotic core, but these areas were excluded from statistical

analysis because the number of areas were low. Sometimes it was difficult to determine whether smooth muscle cells or macrophages expressed the integrin because they commonly co-localized in intimas of carotid arteries. Thus, we simply noted whether the integrin staining was positive in the area. In all cases, there was no doubt as to the negative expression of integrins by the cells. Areas that show positive integrin expression was confirmed by two independent pathologists. It was reassuring that there was no discrepancy between the two pathologists in their opinion of which areas were positive for integrin expression.

Results

In vitro interaction between smooth muscle cell and fibrin

Human vascular smooth muscle cells (HNB18E6E7) were trypsinized and mixed with human plasma. The cells were embedded in plasma clots by addition of thrombin. In the plasma clots, cells were round just after the clot formation (Figure 5.1A); however, spreading of smooth muscle cells was observed by 24 hours (Figure 5.1B) in a time dependent phenomenon (Figure 5.1C). Combination of anti- $\alpha v \beta 3$ (LM609) and anti- $\beta 1$ (P4C10) inhibited cell spreading dramatically (Figure 5.2A). However, neither antibody alone could inhibit spreading even at 50 $\mu\text{g/ml}$ (Figure 5.2B) This was also true when the anti- $\alpha 5 \beta 1$ antibody (JSB5) was used instead of the anti- $\beta 1$ antibody (P4C10) (Figure 5.2B). These two integrins, $\alpha v \beta 3$ and $\alpha 5 \beta 1$, are required for smooth muscle cell spreading in human plasma clots. The cell spreading assay was also performed using RGD peptides. Linear RGD peptides had no effect on spreading. However, GPenGRGDSPCA inhibited cell spreading, suggesting that this peptide blocked $\alpha 5 \beta 1$ as well as $\alpha v \beta 3$ (Figure 5.3A).

In vitro migration into fibrin clots

Migration of human vascular smooth muscle cells was not inhibited by single anti-integrin antibodies (Figure 5.3B). Anti- $\alpha v \beta 3$ (LM609), anti- $\beta 1$ (P4C10) and anti- $\alpha 5 \beta 1$ (JSB5), used at 50 $\mu\text{g/ml}$, inhibited spreading as well as

attachment to relevant substrates such as vitronectin and fibronectin (data not shown). However, a combination of anti- $\alpha v\beta 3$ and anti- $\beta 1$ or anti- $\alpha v\beta 3$ and anti- $\alpha 5\beta 1$ dramatically inhibited migration (Figure 5.3B). This suggests that both $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins on vascular smooth muscle cells are required for migration into fibrin clots.

Western blot analysis

We performed Western blot analysis using two antibodies: #350 that recognizes both fibrin and fibrinogen and T2G1 that is specific for fibrin. Band intensities were measured by a computer assisted system. As a positive control for fibrin we used lysates from a thrombus that was found in an abdominal aortic aneurysm (Figure 5.4, lane 4). The band intensity with #350 was weak in a normal carotid artery obtained from an autopsy patient (Figure 5.4, lane 1), medium in diffuse intimal thickening from a carotid endarterectomy (Figure 5.4, lane 2) and strong in an advanced plaque from an endarterectomy (Figure 5.4, lane 3). This indicates that the total amount of fibrin and fibrinogen is small in normal arteries and larger in advanced plaques. The band intensities of #350 and T2G1 in both advanced plaque (Figure 5.4, lane 3) and thrombus (Figure 5.4, lane 4) were similar, indicating a predominance of fibrin versus fibrinogen in these lesions. However, the intensity of T2G1 was weaker than that of #350 in diffuse intimal thickening (Figure 5.4, lane 2). This suggests that both fibrin and fibrinogen accumulated in this lesion.

Tissue factor and fibrin localization in the intima

To test the specificity of T2G1, immunohistochemistry was first performed using control slides of either fibrin or fibrinogen. Human plasma was placed on slides and allowed to air dry. To some slides, thrombin was applied for five minutes at room temperature to form fibrin. To other slides, PBS was added to form fibrinogen. As shown in Figure 5.5, T2G1 was specific for fibrin and not for fibrinogen. Next, two different areas from one patient were tested (Fig 5.6). One contained a necrotic core (Figure 5.6A) and the other showed diffuse intimal thickening (Figure 5.6B). Fibrin, indicated by positive staining with MAb T2G1, was found in both samples (Figures 5.6C, D). The necrotic core contained heavy fibrin accumulation

(Figure 5.6C) while diffuse intimal thickening had diffuse fibrin accumulation (Figure 5.6D). In the peripheral area of the necrotic core, fibrin was associated with tissue factor (Figure 5.6E). A necrotic core was identified in a total of 34 areas and all were associated with tissue factor expression. However, in this particular case of diffuse intimal thickening, there was no co-localization between fibrin and tissue factor (Figure 5.6F). Overall tissue factor was found in 14 of a total of 27 areas of diffuse intimal thickening. Interestingly, fibrin was always co-localized with macrophages.

Fibrin in the non-atherosclerotic intima of carotid endarterectomy samples

Areas positive for T2G1 as well as #350, were found in all 84 slides. We selected 54 cross sections for quantitative analysis. Positive circumference / total circumference was calculated (Table 5.1). Neither antibody ever stained the media. As expected, fibrin was frequently observed in advanced plaques. However, more than half the circumference of the intima was unexpectedly positive in 13 of the 20 (65%) samples of diffuse intimal thickening. We considered the possibility that the intimal fibrin formation might be due to surgical manipulation performed at the time of the endarterectomy. To eliminate this possibility, we examined four specimens taken from autopsy tissue at the same site and found similar results. In contrast, 5 of the 7 diffuse intimal thickening specimens from coronary arteries of heart transplant recipients were negative for fibrin. Thus, intimal fibrin formation may be a property of the carotid artery.

Integrin expression in diffuse intimal thickening

Diffuse intimal thickening from carotid endarterectomies showed fibrinogen and fibrin accumulation mainly in the intima near the lumen (Figures 5.7A,B). α -actin staining showed smooth muscle cells in superficial and deep intima (Figure 5.7C,D). CD68 staining showed macrophages in the intima (Figure 5.7D). Co-localization between fibrin and macrophage was found, however, tissue factor was not detected on the macrophages (data not shown). Staining by Y2/51, a β 3 integrin antibody, showed β 3 integrin expression by macrophages (Figure 5.7E). However, smooth muscle cells were negative for this antibody. In contrast, staining was positive in all areas including smooth muscle cells, macrophages and endothelial cells

when using the anti- $\beta 1$ antibody, P4C10 (Figure 5.7F). The $\beta 1$ integrin, not the $\beta 3$ integrin, was predominant in the intima. In the 84 slides we studied, $\beta 1$ integrin expression was always greater than $\beta 3$ expression in the intima of carotid endarterectomies.

Integrin expression in intramural hemorrhage.

Figure 5.8 is an area of recent plaque rupture and intramural hemorrhage which is indicated by the presence of sideroblasts in the tissue (data not shown). Staining with T2G1 showed a heavy accumulation of fibrin in the area (Figure 5.8A, 5.8B). α -actin staining indicated the presence of some smooth muscle cells in the intramural hemorrhaged area (Figure 5.8C, 5.8D). $\alpha v\beta 3$ integrin staining was negative on these smooth muscle cells (Figure 5.8E, 5.8F). The $\alpha 5\beta 1$ integrin was expressed on smooth muscle cells in the hemorrhaged area (Figure 5.8G, 5.8H).

Positivity of $\alpha v\beta 3$ integrin in the intima of carotid arteries

Table 5.2 summarizes the frequency of finding areas positive for specific integrins. The $\alpha v\beta 3$ integrin was expressed in only 24% of the areas containing intramural hemorrhage, but $\alpha 5\beta 1$ was positive in 88% of these areas ($p < 0.001$). Expression of $\alpha v\beta 3$ integrin was seen in 55% of the fibrous caps, however, this was less than $\alpha 5\beta 1$ (90%, $p < 0.05$). In regions of diffuse intimal thickening, the frequency of $\alpha v\beta 3$ expression was higher (75%), however, it was still less than $\alpha 5\beta 1$ (97%, $p < 0.05$). In all the intimal areas that we studied, the frequency of $\alpha v\beta 3$ was significantly less than $\alpha 5\beta 1$. Moreover, $\alpha v\beta 3$ was rarely seen in areas of recent plaque rupture.

Discussion

This study shows that $\alpha v\beta 3$ is not, by itself, sufficient to support either migration or spreading of cultured smooth muscle cells in fibrin clots. Moreover, while we have shown that some intimal and plaque smooth muscle cells do express $\alpha v\beta 3$ *in vivo*⁶⁵, we do not see $\alpha v\beta 3$ in human vascular tissue at sites of probable recent injury. These observations suggest

that β_3 antagonists will only be effective on restenosis if platelets are the critical target.

Our focus on intramural fibrin represents a generally unexplored area. There is extensive evidence that high plasma fibrinogen is a risk factor for atherosclerosis¹⁹²⁻¹⁹⁴ and restenosis¹⁸². This hypothesis is supported by several observations including our own finding that an inhibitor of the extrinsic coagulation pathway can block restenosis³⁷. Dvorak *et al.* have suggested that fibrin plays a critical role in tumor angiogenesis, a feature also seen in advanced atherosclerotic plaque¹⁹⁵. A small literature exists about fibrin function in culture systems. Fibroblasts¹⁹⁶, endothelial cells¹¹⁶, melanoma cells¹⁹⁷ and smooth muscle cells¹²⁵ all migrate into fibrin clots and will contract fibrin clots much as they contract collagen clots^{69, 116, 125, 145}. It has been hypothesized that such contraction mechanisms as well as invasion may be important for the interactions of cells with the "provisional wound matrix" around the wound¹⁹⁸⁻²⁰¹ and during wound contraction.

Our studies identify $\alpha_5\beta_1$ and $\alpha\nu\beta_3$ as critical integrins in smooth muscle interactions with fibrin. Five integrins have been shown to bind fibrin or fibrinogen: $\alpha_M\beta_2$ ²⁰², $\alpha_{IIb}\beta_3$ ²⁰³, $\alpha\nu\beta_3$ ¹⁸⁰, $\alpha\nu\beta_1$ ⁸¹ and $\alpha_5\beta_1$ integrins⁸³. The $\alpha_M\beta_2$ integrin is expressed on leukocytes and $\alpha_{IIb}\beta_3$ is expressed on platelets. The $\alpha\nu\beta_1$ integrin has been described as an alternative receptor for fibrin on melanoma cells which do not express $\alpha\nu\beta_3$ ⁸¹. In melanoma cells, $\alpha\nu\beta_3$ has been shown to bind fibrin or fibrinogen¹⁸⁰. Endothelial cells expressing $\alpha_5\beta_1$ bind fibrinogen in the presence of manganese⁸³. Therefore, smooth muscle cells could interact with fibrinogen through $\alpha\nu\beta_3$ ¹⁸⁰, $\alpha\nu\beta_1$ ⁸¹ or $\alpha_5\beta_1$ integrins⁸³. We have previously shown that $\alpha_5\beta_1$ is critical for fibrin clot contraction¹²⁵. Both $\alpha\nu\beta_3$ and $\alpha_5\beta_1$ are responsible for transmigration of fibroblasts from collagen into fibrin²⁰⁴. In intimal formation at the ductus arteriosus, both $\alpha\nu\beta_3$ and $\alpha_5\beta_1$ were up-regulated on smooth muscle cells at the newly formed intima in monkeys²⁰⁵. Similarly, the present study shows that two integrins, $\alpha_5\beta_1$ and $\alpha\nu\beta_3$, are required for *in vitro* interaction between human vascular smooth muscle and fibrin.

In vitro functional assays, however, can not be interpreted unless we also know the target molecules that are present at the sites of interest. For example, despite numerous *in vitro* studies implicating $\alpha 2\beta 1$ in smooth muscle cell interaction with collagen^{64, 206}, this integrin is only seen on the endothelium in the vessel wall and never seen on smooth muscle cells except during development²⁰⁵. In a previous report, we found the expression of $\alpha v\beta 3$ integrin on intimal cells *in vivo*⁶⁵. The intima is usually assumed to be the layer critical for vessel response to injury. However, in our previous report, $\beta 1$ was universally present while $\beta 3$ was seen only in a small portion of the intimal smooth muscle cells. Similarly, in the present study, $\alpha v\beta 3$ integrin was rarely seen on smooth muscle cells even in areas of recent injury identified by evidence of intramural hemorrhage. Geary *et al.* have shown similar results. $\alpha v\beta 3$ was expressed less in intima than in media in a monkey vascular injury model³⁹. When $\alpha v\beta 3$ was seen in lesions, it was usually focally located on macrophage or endothelial cells.

These data are not inconsistent with animal studies showing prevention of intimal hyperplasia with cyclic RGD peptides^{150, 152, 207}. It is important to note that the peptides are not specific for only $\alpha v\beta 3$ ^{162, 166, 206, 208}. For example, the assertion that GPenGRGDSPCA is specific to $\alpha v\beta 3$ was based on studies of normal rat kidney cells⁹⁴. The GPenGRGDSPCA was unable to inhibit cell adhesion to fibronectin at under 1 mM although a little inhibition was seen at over 1 mM⁹⁴. However, we have found that the GPenGRGDSPCA blocks the attachment to fibronectin as well as vitronectin using human vascular smooth muscle cells at lower concentration (K. Yee, unpublished data, 1998). We have also shown here that GPenGRGDSPCA blocked both $\alpha 5\beta 1$ and $\alpha v\beta 3$ in spreading assay of human vascular smooth muscle cells.

Another possibility is that the critical matrix molecule supporting migration at sites of injury is collagen. However, *in vivo*, $\alpha 2\beta 1$, a known collagen receptor, is apparently only expressed on smooth muscle cells before birth²⁰⁵. Studies analyzing the function of integrins during smooth muscle cell interaction with collagen can not be interpreted because expression of $\alpha 2\beta 1$ on smooth muscle cells is an *in vitro* artifact. This

expression may overwhelm any normal adherence and signaling pathways that exist.

We need to conclude by commenting on the serendipitous discovery in this study of finding fibrin in non-atherosclerotic intimas of carotid arteries. Observation of fibrin in advanced lesions are not new. Bini *et al*, using antibodies similar to those employed here, showed that fibrin was prominent in the advanced lesions of aortas or coronary arteries but that diffuse intimal thickening mainly showed fibrinogen¹⁸⁴. Valenzuela *et al*, in contrast, reported that fibrin was prominent in the pseudo-intima of prosthetic devices while fibrinogen preferentially accumulated in the atherosclerotic aortic plaque⁴⁸. Thus, we might have expected arterial intima to contain fibrinogen. However, the presence of fibrin in the intima of the carotid arteries but not in the coronaries suggests that the carotid intima may express tissue factor, the usual requisite to initiate coagulation²⁰⁹⁻²¹¹. Though we did find a high frequency of CD68 positive cells, macrophages, in diffuse intimal thickening in the carotid artery, about half of the diffuse intimal thickening containing fibrin did not stain for tissue factor. Thus, one must posit either that coagulation in these sites is being promoted by some pathway other than the extrinsic pathway or that the time of active coagulation has passed when we sampled the tissues. This may reflect episodes of local inflammation and activation of tissue factor in "normal" intima, as proposed by Libby for plaques²¹². Our data suggest that fibrin is much more wide spread in the arterial intima than previously suspected. This fibrin could play a key role in vascular narrowing, as suggested by the effect of a blocker of the extrinsic cascade³⁷.

In summary, we suggest that more attention should be paid to the possible biological role that intramural fibrin plays during the response to injuries produced by both spontaneous rupture and angioplasty. In addition to our own focus on vascular remodeling, other possibilities for fibrin include its role in intima-plaque angiogenesis in tumors as suggested by Dvorak¹⁹⁵. The integrins controlling this pathway could be intriguing clinical targets.

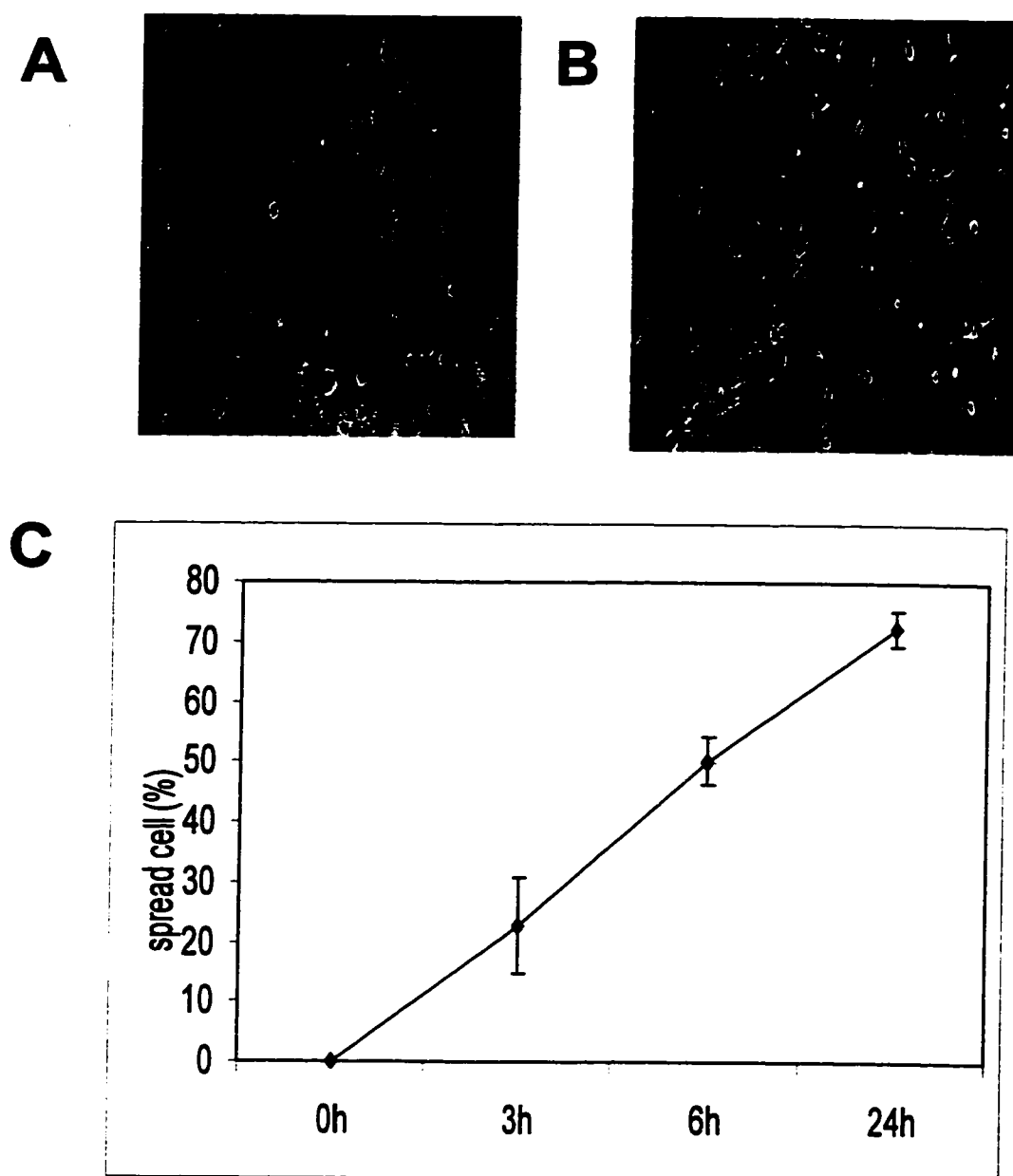


Figure 5.1. Cell spreading in human plasma clots.

Human vascular smooth muscle cells (HNB18E6E7) were embedded in human plasma clots. The clot was incubated at 37°C for 24 hours. (A) just after clot formation (t=0), (B) 24 hours after incubation (t=24h). (C) Time course of cell spreading. Error bars represent \pm standard deviation.

Figure 5.2. Inhibition of cell spreading in plasma clots by a combination of monoclonal anti-integrin antibodies.

(A) The same protocol was followed as described in figure 5.1 except that cell spreading was inhibited by a combination of anti- $\alpha v\beta 3$ (LM609) and anti- $\beta 1$ (P4C10) antibodies. The x axis represents the total antibody concentration. Therefore, 50 $\mu\text{g/ml}$ is equivalent to 25 $\mu\text{g/ml}$ of LM609 plus 25 $\mu\text{g/ml}$ of P4C10. Control samples were non-immune mouse IgG at the same concentrations. Error bars represent \pm standard deviation. **(B)** Human smooth muscle cells were preincubated with the following antibodies: LM609 (anti- $\alpha v\beta 3$), P4C10 (anti- $\beta 1$) or JBS5 (anti- $\alpha 5\beta 1$). The antibodies were added singularly or in combination. The antibody concentrations were 50 $\mu\text{g/ml}$. The same experimental procedure was followed as described in figure 5.1. Error bars represent \pm standard deviation.

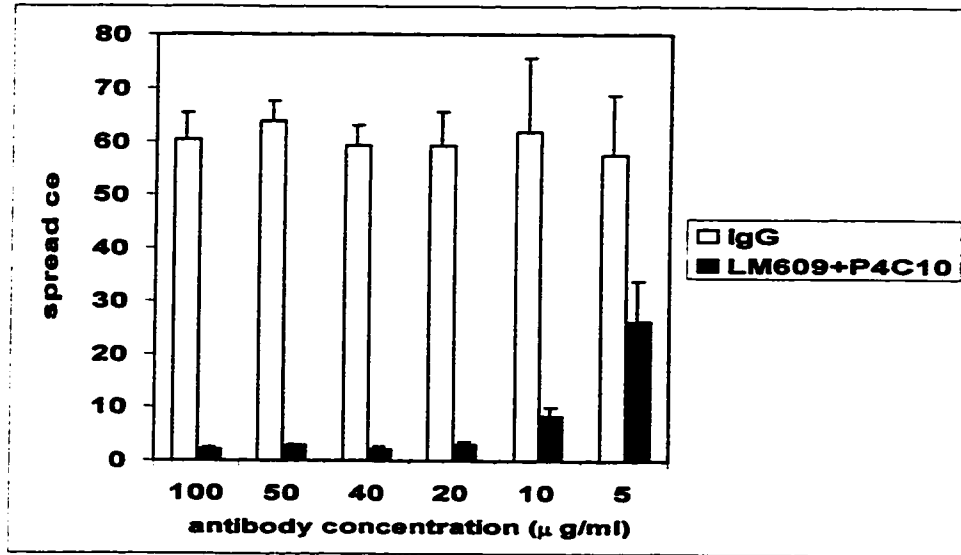
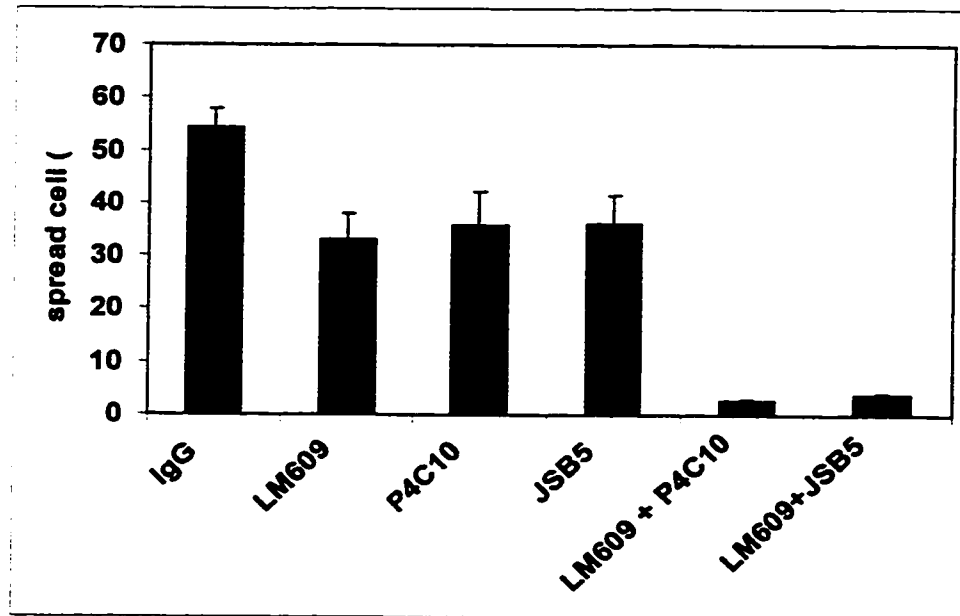
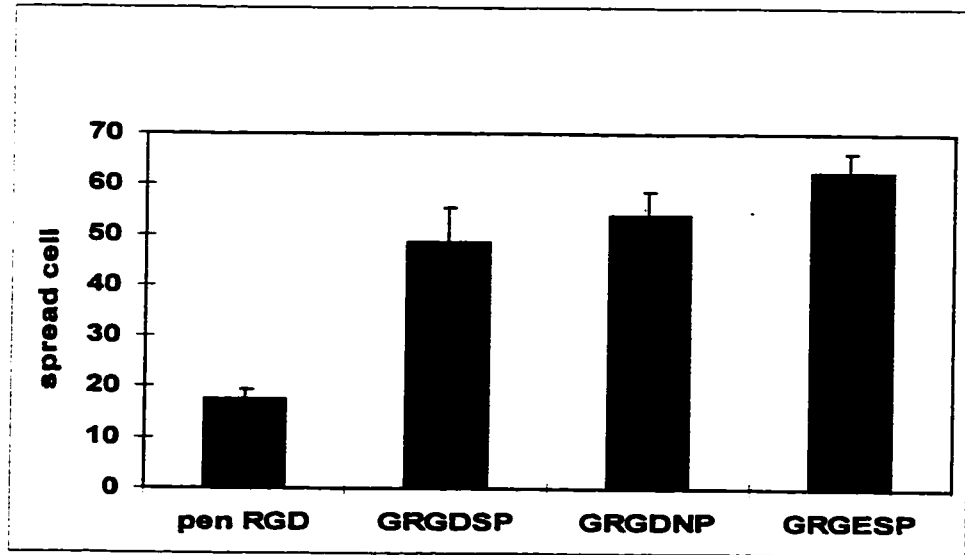
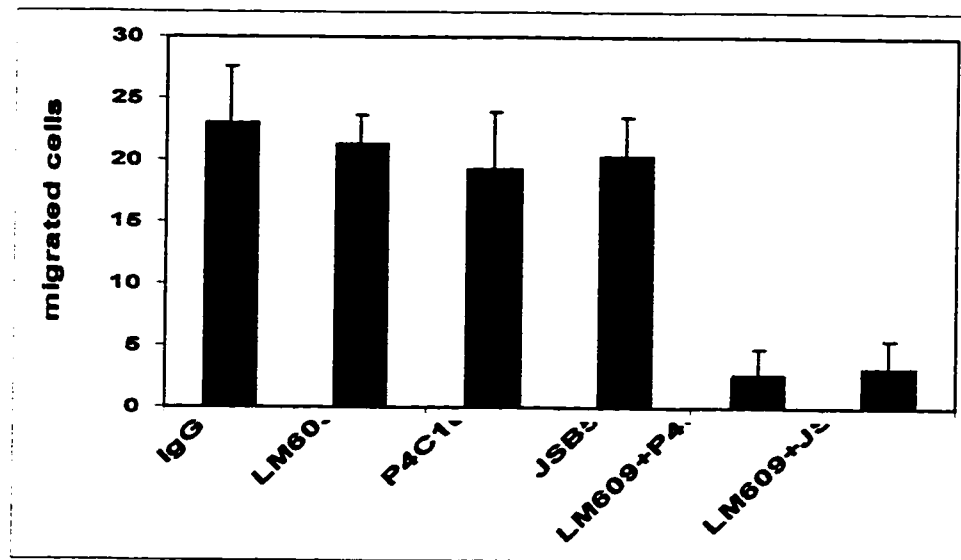
A**B**

Figure 5.3A. Inhibition of cell spreading in plasma clots by cyclic RGD peptides

The same experimental procedure was followed as described in figure 5.3A except that the human smooth muscle cells were preincubated with 500 μM of linear and cyclic RGD (GPenGRGDSPCA) peptides. Error bars represent \pm standard deviation.

Figure 5.3B. Invasion and migration of human smooth muscle cells into fibrin clots.

The invasion/migration assay into fibrin clots was performed using a modification of the method originally reported by Naito *et al* (see material and methods). Inhibition of migration by the following antibodies at 20 $\mu\text{g}/\text{ml}$ was not observed: LM609 (anti- $\alpha\text{v}\beta\text{3}$ integrin), P4C10 (anti- β1 integrin) or JSB5 (anti- $\alpha\text{5}\beta\text{1}$ integrin). However, a combination of LM609 and P4C10 dramatically inhibited migration ($p < 1 \times 10^{-10}$). The extent of inhibition seen by combining LM609 and JSB5 was similar ($p < 1 \times 10^{-10}$). The bars represent mean \pm standard deviation. Statistical analysis was done using chi-square test.

A**B**

Normal DIT Plaque Thrombus

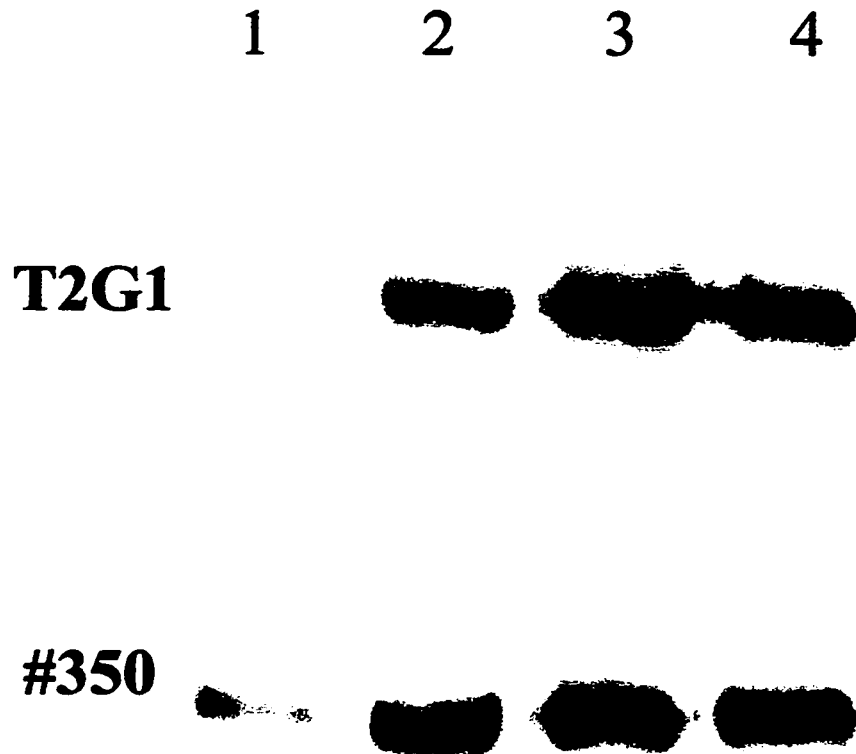


Figure 5.4. Western Blot Analysis using MAb #350 and MAb T2G1 on reduced carotid artery samples.

Lysates from normal carotid artery (lane 1), diffuse intimal thickening (lane 2), advanced plaque (lane 3) and thrombus from an abdominal aortic aneurysm (lane 4) were electrophoresed through an 8% SDS polyacrylamide gel, transferred to a PVDF membrane and probed with either #350 or T2G1.

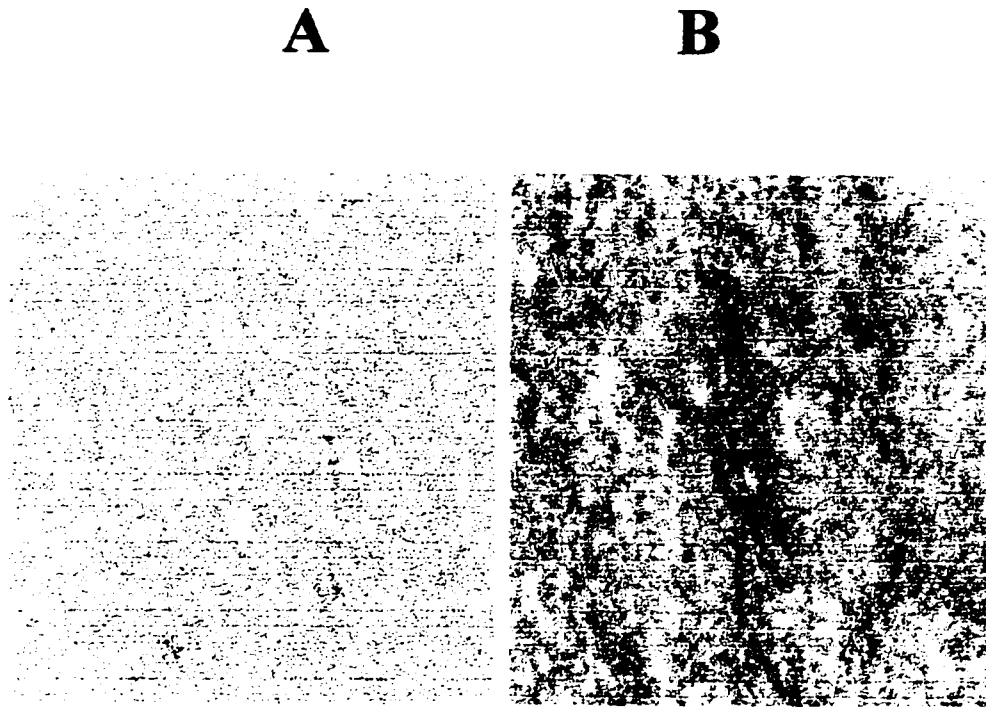


Figure 5.5. Specificity of MAb T2G1.

Human plasma was air-dried on slides and either PBS (A) or thrombin (B) was added to the slides. Immunohistochemistry was performed on the samples using monoclonal antibody 2TG1.

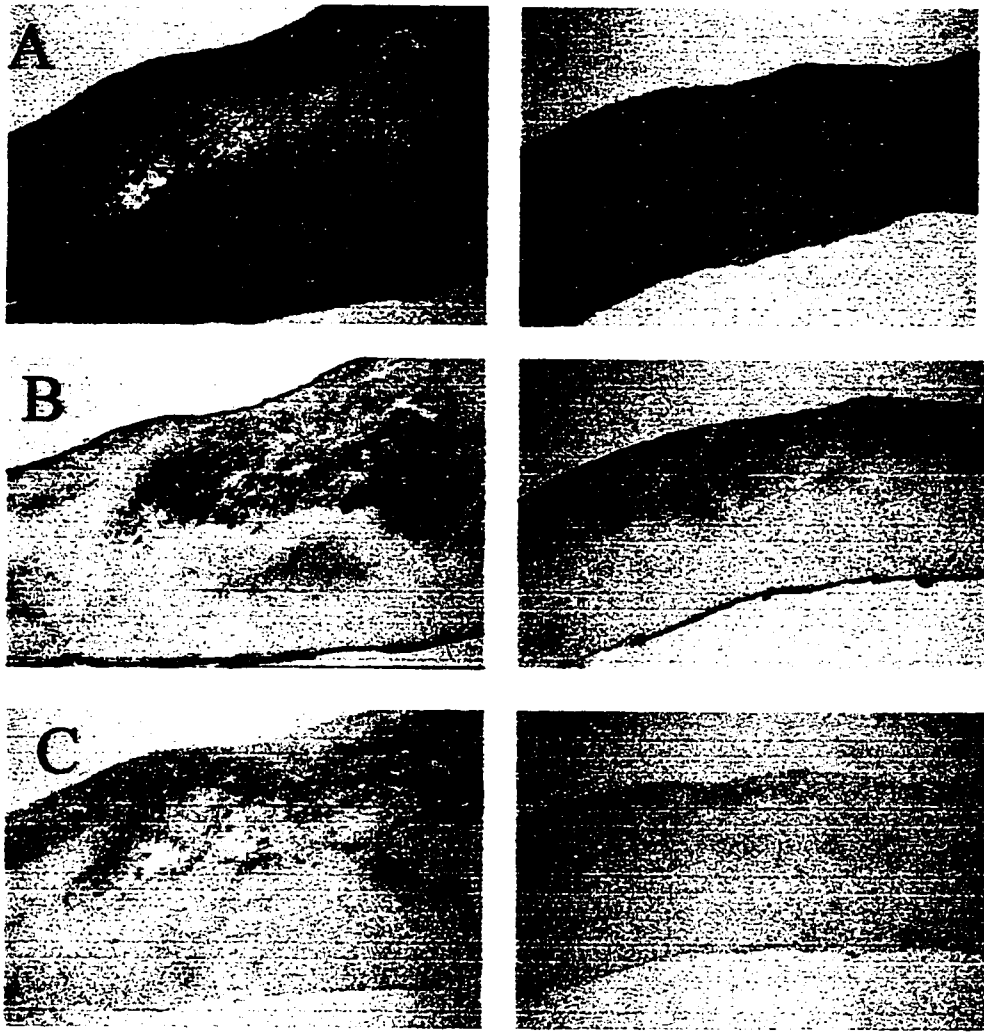


Figure 5.6. Expression of tissue factor and fibrin in carotid endarterectomy samples.

These samples were derived from two separate areas from one patient's carotid artery. In the first sample, H&E staining reveals the necrotic core in the middle of the intima (A). Fibrin stained by MAb T2G1 is positive at the necrotic core (B) and tissue factor is also positive at the peripheral area of the necrotic core (C). In the second sample, H&E staining shows existence of diffuse intimal thickening in the tissue (D). Fibrin stained with MAb T2G1 is positive in the intima near the lumen (E). However, tissue factor does not co-localize with fibrin (F). (Original magnification was 4X.)

Figure 5.7. Integrin expression in diffuse intimal thickening.

The lumen is located at the left top of the pictures. This endarterectomy sample has only a thin layer of media. Samples of diffuse intimal thickening were stained for fibrinogen with MAb #350 (A) and for fibrin with MAb T2G1 (B). Both fibrin and fibrinogen are found in the intima near the lumen. α -actin staining indicates that smooth muscle cells are present in the intima and the media (C). CD68 staining reveals that macrophages are found in the intima (D). In this case, macrophages are co-localized with fibrin, however, these macrophages do not express tissue factor (data not shown). Staining with Y2/51 for $\beta 3$ integrin shows positive $\beta 3$ expression on macrophages located at the right middle field where there are no smooth muscle cells present (E). Staining with MAb P4C10, an anti- $\beta 1$ integrin antibody, is positive in all areas of the tissue including on smooth muscle cells, macrophages and endothelial cells (F). $\beta 1$ integrin is a predominant integrin in the intima. (Original magnification is 4X).

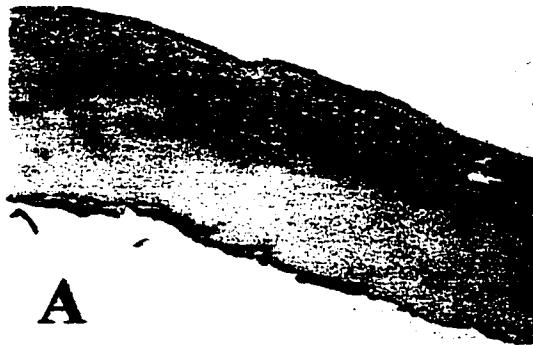


Figure 5.8. Integrin expression in areas of intramural hemorrhage from an endarterectomy sample.

This lesion contains an intramural hemorrhage caused by recent intimal rupture. Panels A and B were stained with MAb T2G1, an anti-fibrin antibody. Heavy fibrin accumulation is seen at the site of intramural hemorrhage (A, 4X; B, 20X). A serial section was stained with an anti- α -actin antibody which identifies smooth muscle cells in the tissue (C, D). Some smooth muscle cells remain in the cap near the lumen and some are in the intramural hemorrhage area (C, 4X; D, 20X). The cells in the hemorrhage may have migrated into the fibrin matrix from the adjacent intima (D, 20X). Staining is negative for the anti- α v β 3 integrin antibody LM609 on these smooth muscle cells (E, 4X; F, 20X). However, it is positive on macrophages in the necrotic core, on vaso vasorum endothelium and on smooth muscle cells in the media. α 5 β 1 integrin, identified by the antibody JSB5, is expressed on both smooth muscle cells and macrophages in the hemorrhagic area (G, 4X; H, 20X).

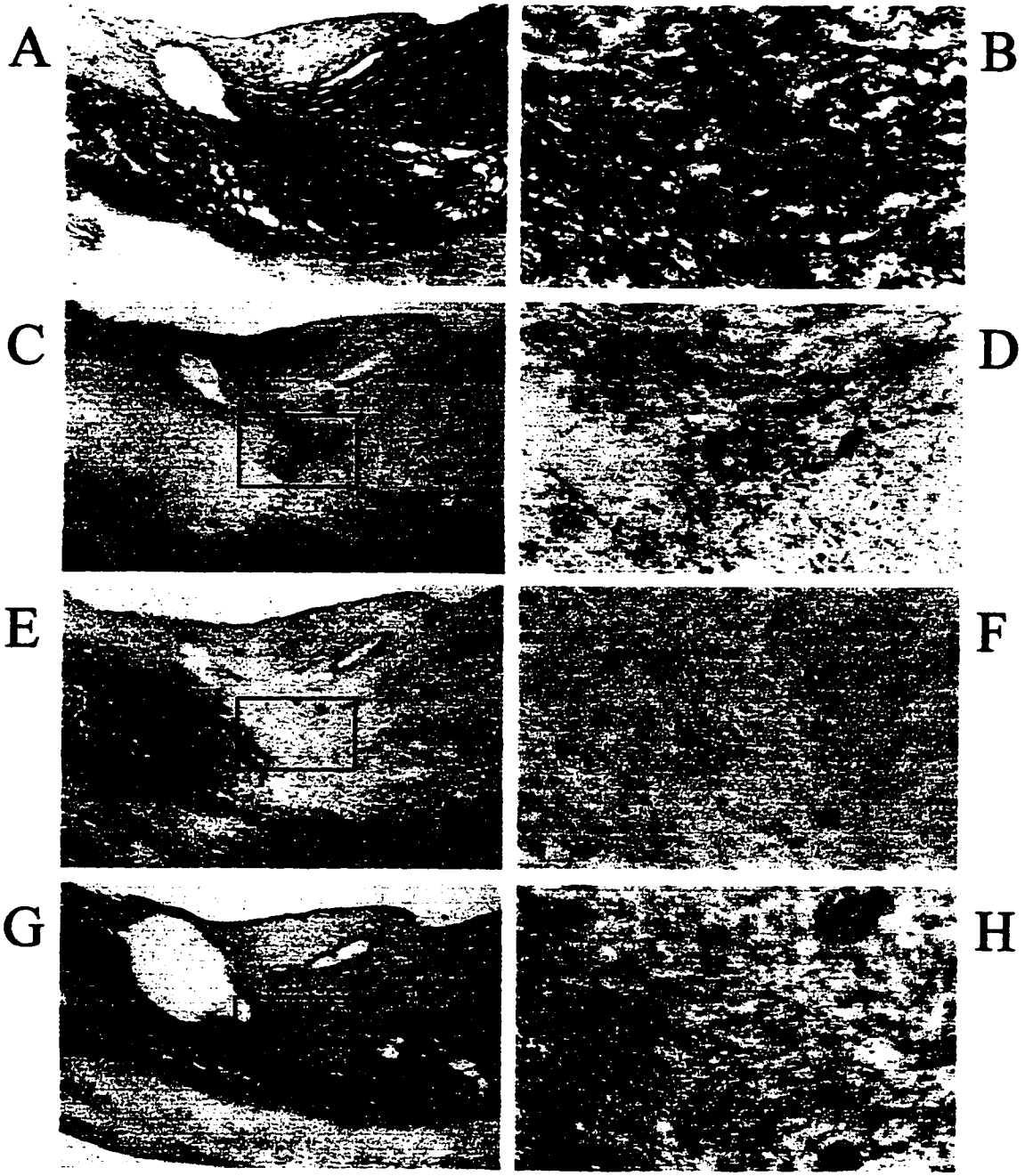


Table 5.1. Fibrin staining of diffuse intimal thickening and plaques from coronary and carotid arteries with T2G1

| Positive/Total Circumference Stained with T2G1 | Coronary | Carotid | |
|--|--------------------------------------|---------------------------------------|----------------|
| | Diffuse Intimal Thickening n=7 | Diffuse Intimal Thickening n=20 | Plaque n=34 |
| 0% | 5 (71%) | 0 | 0 |
| 1%-5% | 2 (29%) | 7 (35%) | 2 (6%) |
| 51-99% | 0 | 9 (45%) | 15 (44%) |
| 100% | 0 | 4 (20%) | 17 (50%) |

Diffuse Intimal Thickening was defined as areas of diffuse thickening without findings of advanced atherosclerosis like necrotic cores. Plaque was defined as intima that contained a fatty mass. Specimens were selected that consisted entirely of cross-sectional plaque or entirely of cross sectional diffuse intimal thickening. No specimens were found that did not contain areas of T2G1 staining except specimens showing no or minimal intimal thickenings in carotid arteries. However, diffuse intimal thickening in coronary arteries from heart transplant recipient contained less fibrin than carotid arteries.

Table 5.2. Distribution of integrins in areas where smooth muscle cells and fibrin were both found.

| Histology | Integrin | + | +/- | - | P-value |
|--|--------------------|----------|---------|---------|---------|
| Hemorrhage n=25 | $\alpha v \beta 3$ | 6 (24%) | 1 (4%) | 18 (7%) | P<0.001 |
| | $\alpha 5 \beta 1$ | 22 (88%) | 2 (8%) | 1 (4%) | |
| Fibrous Cap n=20 | $\alpha v \beta 3$ | 11 (55%) | 4 (20%) | 5 (25%) | P<0.05 |
| | $\alpha 5 \beta 1$ | 18 (90%) | 1 (5%) | 1 (5%) | |
| Diffuse Intimal Thickening n=36 | $\alpha v \beta 3$ | 24 (67%) | 3 (8%) | 9 (25%) | P<0.05 |
| | $\alpha 5 \beta 1$ | 35 (97%) | 1 (3%) | 0 | |

Hemorrhage was defined as areas containing red cells and sideroblasts. Fibrous cap was defined as a fibrous capsule separating the lumen from the vessel wall. Diffuse intimal thickening was defined as areas of diffuse thickening without findings of advanced atherosclerosis like necrotic cores. Frequency values were set to the incidence of areas showing the features listed. For example, of 25 areas of intramural hemorrhage on 20 slides, 6 areas contained cells that stained for $\alpha v \beta 3$. P-value was calculated by chi-square test comparing $\alpha v \beta 3$ integrin with $\alpha 5 \beta 1$ integrin.

Chapter 6: Discussion and Future Directions

Cell-matrix interactions are important components of many biological activities such as development, wound healing and disease progression. One disease of interest is atherosclerosis. Narrowing of the large, muscular arteries or rupture of the atherosclerotic lesion is one of the primary causes of morbidity and mortality in humans. To assist in finding treatments for this disease, many researchers have attempted to identify molecules that could serve as targets for pharmaceutical intervention. This thesis arises from the hypothesis that wound healing is a mechanism of vessel narrowing during advanced stages of the disease. We have suggested that fibrin, an extracellular matrix found in all wounds and a prominent feature of lesions, could serve as a focal point for cellular interaction and decrease the cross-sectional area of the vessel lumen.

In this dissertation, I have characterized how smooth muscle cells interact with fibrin in order to identify molecules that would serve as targets for pharmaceutical development. In summary my studies have demonstrated that human smooth muscle cells are able to interact with a fibrin clot composed of either recombinant fibrinogen, gelatin sepharose treated plasma or whole plasma. By using antibodies against various members of the integrin receptor family, I have identified two integrins that are important for smooth muscle cell adhesion, contraction and migration on fibrin clots. One integrin, $\alpha 5\beta 1$, seems to play a greater role than the other, $\alpha v\beta 3$, in human smooth muscle cell interaction with fibrin. I have also demonstrated that fibronectin, a matrix molecule found in plasma and secreted by cells, acts as a mediator between smooth muscle cells and the fibrin matrix. Fibronectin binds to fibrinogen and is the ligand for the integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$. This model has physiological relevance *in vivo* since $\alpha 5\beta 1$, fibronectin and smooth muscle cells are found in atherosclerotic plaques.

Adhesion on recombinant fibrin clots

Smooth muscle cell adhesion to recombinant fibrin is mediated by the $\alpha 5\beta 1$ integrin on the smooth muscle cell and hamster fibronectin found in recombinant fibrinogen. Antibodies against the $\beta 1$ integrin (P4C10), $\alpha 5$ integrin subunit (Mab 16), $\alpha 5\beta 1$ (JBS5) integrin and fibronectin (R790) inhibited cell adhesion to recombinant fibrin. Furthermore, the peptides

penRGD, VCAM345 and G4120 also inhibited cell adhesion to the matrix. A disintegrin, Kistrin, had the most potent effect on inhibiting cell adhesion to recombinant fibrin.

In this two-dimensional assay system, smooth muscle cells adhere to recombinant fibrin that has been coated onto plastic. In this situation, cells are interacting with insoluble fibronectin in the fibrin matrix and they utilize the integrin $\alpha 5\beta 1$ to attach to this matrix. Even though $\alpha v\beta 3$ is also a receptor for fibronectin, its role is small due to the low numbers of receptors on the smooth muscle cell surface. So it is not surprising that LM609, an anti- $\alpha v\beta 3$ antibody, only partially blocked cell adhesion. However, penRGD and G4120, $\alpha v\beta 3$ peptide antagonists, significantly blocked cell adhesion to this matrix. Even though penRGD has been labeled an αv inhibitor, our data demonstrate that it also can inhibit smooth muscle cell adhesion to fibronectin. Because the low number of $\alpha v\beta 3$ on the cell surface can not account for all the cell-fibronectin interaction, penRGD must also inhibit $\alpha 5\beta 1$ interaction with fibronectin. In agreement with our data, penRGD was more effective than G4120 in inhibiting smooth muscle cell adhesion to recombinant fibrin. G4120 inhibited cell adhesion to vitronectin better than penRGD, but it was not as effective in inhibiting cell adhesion to fibronectin. This suggests that G4120 is more specific for $\alpha v\beta 3$ than penRGD is for this integrin. Indeed, its effect on inhibiting adhesion to recombinant fibrin was comparable to LM609, an antibody against $\alpha v\beta 3$.

Adhesion on gelatin sepharose treated plasma clots and whole plasma clots

Smooth muscle cell adhesion to gelatin sepharose treated plasma clots involves the integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$, as well as the matrix proteins fibronectin and vitronectin. Antibodies against $\alpha v\beta 3$ (LM609), αv (L230), $\alpha 5\beta 1$ (JBS5), $\alpha 5$ (Mab 16), $\beta 1$ (P4C10), fibronectin (R790) and vitronectin inhibited smooth muscle cell adhesion to gelatin sepharose treated plasma clots. Antibodies against $\alpha v\beta 3$ were more effective than those against $\alpha 5\beta 1$ in inhibiting cell adhesion suggesting a greater role for $\alpha v\beta 3$ in smooth muscle cell adhesion to this matrix. This was confirmed when an antibody against vitronectin inhibited cell adhesion. Data on the effectiveness of the peptides penRGD, G4120 and VCAM345 to inhibit cell adhesion also support this conclusion. VCAM345, an anti- $\alpha 4$ and anti- $\alpha 5$ peptide antagonist, was

not as effective as the $\alpha v\beta 3$ peptide antagonists, G4120 and penRGD, in inhibiting cell adhesion. As in the case of recombinant fibrin, the disintegrin Kistrin had a very potent effect in preventing smooth muscle cell adhesion to gelatin sepharose treated plasma clots. The data suggest that smooth muscle cells are adhering to this matrix through the integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ with $\alpha v\beta 3$ playing a greater role due to the presence of vitronectin. The plasma protein fibronectin is also important in this interaction. Western blot analysis demonstrates the presence of low molecular weight fragments of fibronectin in this source of fibrinogen. These small fibronectin fragments may not be as effective as the high molecular weight fibronectin in promoting cell adhesion.

Adhesion to whole plasma clots represents a more physiological system because whole plasma contains almost all of the proteins that would be found in a clot *in vivo*. Smooth muscle cell adhesion to whole plasma clots was inhibited by the disintegrin Kistrin and the anti-fibronectin antibody R790 but not by the cyclic peptides VCAM345, penRGD and G4120. The data suggest that smooth muscle cell adhesion to whole plasma clots is mediated by fibronectin found in the whole plasma clots and by several integrins, possibly $\alpha 5\beta 1$ and $\alpha v\beta 3$. Kistrin, a pan-integrin antagonist was the most effective in inhibiting cell adhesion to whole plasma clots at low concentrations. The data indicate that cell adhesion to this matrix involves multiple integrins due to the complexity of proteins in the clot. To effectively block all receptor-ligand interactions, all the receptors must be blocked or all the ligands must be blocked. This data is supported by the fact that a high concentration of Kistrin was needed to block cell adhesion to this matrix.

Contraction of Fibrin Clots

Contraction of recombinant fibrin clots, gelatin sepharose treated plasma clots, and whole plasma clots by human smooth muscle cells is mediated by the $\alpha 5\beta 1$ integrin and the molecule fibronectin. In all three cases only the anti- $\beta 1$ antibody (P4C10), anti- $\alpha 5$ antibody (Mab 15), anti- $\alpha 5\beta 1$ antibody (JBS5), anti-fibronectin antibody (R790) and the disintegrin Kistrin inhibited contraction of fibrin clots. Antibodies and peptides against αv and $\alpha v\beta 3$ were generally not effective (L230, LM609, 7E3 Fab, penRGD, and

G4120). Furthermore, the anti- α 4 and anti- α 5 peptide antagonist, VCAM345, was also not able to inhibit clot contraction. However, penRGD, at a concentration between 250-500 μ M, was able to block clot contraction of gelatin sepharose treated plasma clots. This concentration range affects cell binding to fibronectin and vitronectin. By blocking both low molecular weight fibronectin and vitronectin, contraction of gelatin sepharose treated plasma clots was inhibited. The effectiveness of Kistrin, a pan-integrin antagonist suggest that multiple integrins are involved in clot contraction with α 5 β 1 playing a dominant role. As explained later, the integrins are not only mediating cell-matrix interactions, but also fibronectin assembly.

Migration on Fibrin Clots

Two assays were used to test smooth muscle cell migration towards and within fibrin clots. The first assay, a Transwell migration assay, tested the cells' ability to migrate through a recombinant fibrin, a gelatin sepharose treated plasma clot or a whole plasma clot. In all three sources of fibrinogen, migration towards and within the fibrin clot was mediated by both the α v β 3 and α 5 β 1 integrins. Antibodies to each of these integrin alone did not inhibit migration but in combination they halted cell migration (data not shown). In support of this statement, the pan-integrin antagonist, Kistrin, was very effective in inhibiting smooth muscle cell migration on fibrin.

The second assay, an invasion/migration assay, demonstrated that smooth muscle cells sandwiched between two layers of fibrin were able to migrate up into the top fibrin layer. This migration into fibrin clots was also dependent on both the α v β 3 and α 5 β 1 integrins as antibodies against both of these integrins were effective in inhibiting migration into the fibrin clot. The role of fibronectin was not explored. However, extrapolation from the adhesion and contraction data predicts that migration would depend on fibronectin.

Fibronectin Incorporation into a fibrin clot

Cell adhesion to recombinant fibrin, gelatin sepharose treated plasma clots and whole plasma clots was inhibited by treating the matrices with an anti-fibronectin antibody. Furthermore, recombinant fibrin clot contraction by smooth muscle cells was statistically inhibited only when the matrix was

pre-incubated with the anti-fibronectin antibody. When the cells or the cells plus the matrix were treated with the antibody, clot contraction was inhibited but it wasn't statistically significant. This would suggest that in these specific situations fibronectin found in the fibrin clots is more important than cell surface fibronectin.

In these assays, smooth muscle cells are interacting with an insoluble matrix of fibrin. Fibrinogen, normally soluble in solution, is rendered insoluble by the cleavage of fibrinopeptides A and B off the α and β chains of fibrinogen, respectively. The de novo sites cause the fibrinogen molecules to interact and Factor XIIIa or tissue transglutaminase cross-links the fibers to form a rigid meshwork. At the same time, soluble fibronectin can also be cross-linked and incorporated into the fibrin matrix by Factor XIIIa²¹³. This could be the situation for gelatin sepharose treated plasma and whole plasma during clot formation in the 96 well plates used for the adhesion assay.

Soluble fibronectin can also be rendered insoluble if it is interacting with the fibrinogen molecule non covalently before thrombin cleavage. When fibrinogen is cleaved and forms a matrix, the fibronectin that is already attached to the fibrinogen molecule could become insoluble with the fibrin matrix. This process may occur during the recombinant fibrin clot contraction assay and adhesion assay. Our initial results that $\alpha 5\beta 1$ mediated cell contraction and adhesion of recombinant fibrin clots were surprising because we assumed that recombinant fibrinogen did not contain fibronectin and that $\alpha 5\beta 1$ could not bind to fibrin. Our later results on the ability of the anti-fibronectin antibody R790 to inhibit cell interaction with recombinant fibrin caused us to search for the source of fibronectin found in recombinant fibrinogen. Through western blot analysis and an ELISA assay, a non-human fibronectin was detected in the recombinant fibrinogen. We hypothesize that during production of recombinant fibrinogen in the hamster chinese ovary cells, fibronectin could be attached to the fibrinogen molecule and secreted from the cell as a unit. During polymerization of the fibrin clot, the fibronectin could be incorporated in the fibrin strands and made available for cellular interaction.

Western blot analysis detected both low and high molecular weight fibronectins in whole plasma, gelatin sepharose treated plasma and

recombinant fibrinogen. Fibronectin is usually found as a dimer in plasma and as multimers on the cell surface. Binding of the 70kDa N-terminal fragment of fibronectin with a fragment of the first type III repeat (III₁+C) can cause fibronectin to aggregate in solution^{214, 215}. The aggregated fibronectin can bind to the fibrin clot or can be cross-linked to the clot by transglutaminases. At this time, it is not known whether any of the fibronectin fragments in plasma or recombinant fibrinogen include III₁+C.

Recently one group explored the effect of the fibronectin fragment III₁+C on vascular smooth muscle cell growth and fibronectin matrix assembly²¹⁶. The authors found that cells plated on fibronectin will proliferate better than cells grown on plastic. However, when the cells were grown on III₁+C, incorporation of labeled thymidine was much lower. This decrease in cell proliferation was specific for rat and human smooth muscles as other cell types, such as endothelial cells, were not affected by fragment III₁+C. Furthermore, fragment III₁+C did not affect $\alpha 5\beta 1$ localization to focal contacts, but it did affect the ability of the cells to assemble a fibronectin matrix. So while III₁+C in solution can cause fibronectin to aggregate, on the cell surface it blocks fibronectin matrix assembly.

Cell Surface Fibronectin Assembly

When we pre-incubated either gelatin sepharose treated plasma or whole plasma with the anti-fibronectin antibody, R790, inhibition of clot contraction did not occur. However, when the cells or the cells plus the matrix were treated with R790, contraction was significantly inhibited. This would suggest that clot contraction of these two matrices relied mainly on cellular fibronectin interaction with the fibrin matrix.

There are regions on the cell surface, called LAMMs (molecules of large apparent molecular mass), that are sites of fibronectin assembly. Fibronectin²¹⁷ assembly requires both the participation of integrins, such as $\alpha 5\beta 1$ and $\alpha v\beta 3$, as well as certain regions of fibronectin: the amino terminal 70-kDa region, the cell adhesion site on the tenth Type III repeat (RGD sequence) and the I9 site on the first Type III repeat. A third inducer of fibronectin assembly is the formation of actin stress fibers in the cell. One of the reagents that induces tension on the cell, lysophosphatidic acid, also increases binding of soluble fibronectin²¹³. Recently Zhong et al.

demonstrated that lysophosphatidic acid modulated its effect through Rho GTPase. In the assays described in this dissertation, smooth muscle cell spreading, contraction and migration on fibrin could increase fibronectin on the cell surface.

Integrins are also key components of matrix assembly on the cell surface. Integrin interaction with the RGD motif on fibronectin is required for fibronectin assembly²¹⁸. $\alpha 5\beta 1$ has been shown to be the principle integrin in fibronectin assembly; however, $\alpha v\beta 3$ can also assemble the matrix on the cell surface in the absence of $\alpha 5\beta 1$ ²¹⁹. Furthermore antibodies against $\alpha 5\beta 1$ inhibited matrix assembly²¹⁸. In our case, anti- $\alpha 5$, $\alpha 5\beta 1$ and $\beta 1$ antibodies may be inhibiting not only integrin-ligand interactions but also fibronectin assembly. It's interesting that the linear RGD peptides did not have an effect on smooth muscle cell interaction with fibrin. Perhaps it's because the RGD peptides were interacting with the cell and increasing fibronectin assembly on the cell surface and this would not decrease clot contraction.

Cell Spreading: Role of Fibronectin

In the course of these studies, it was noted that smooth muscle cells would spread when adhering to gelatin sepharose treated plasma clots or whole plasma clots coated on plastic or in a three dimensional plasma clot. However, when smooth muscle cells were immersed in a recombinant fibrin clot (three dimensional), they did not spread and while adhering to recombinant fibrin coated on plastic, they slightly spread. Cell adhesion to any matrix does not require cell spreading, a process involving the cytoskeleton. We initially postulated that the difference in cell shape was due to a lack of either fibronectin or vitronectin in the recombinant fibrin clot. That is to say that cells will not spread on pure fibrin. However, we now have evidence that in recombinant fibrin, there is hamster fibronectin present, though this may not be enough to promote cell spreading. The other difference between plasma and recombinant fibrinogen is the presence of Factor XIIIa, a transglutaminase. Perhaps the combination of fibronectin, fibrin and factor XIIIa is conducive for cell spreading.

Summary: Difference Between Plasma and Recombinant Fibrinogen

Smooth muscle cell adhesion to whole plasma clots is mediated by both fibronectin and vitronectin in the plasma. The integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ are the receptors for these molecules, respectively. The three ligands allow the cells to maintain cell adhesion if one of the integrins is blocked. However, because there are more $\alpha 5\beta 1$ integrins than $\alpha v\beta 3$ integrins, blocking this integrin will have a greater effect than blocking the $\alpha v\beta 3$. However, if you block both integrins by using Kistrin, adhesion is nearly abolished. The same is true for smooth muscle cell adhesion to gelatin sepharose treated plasma clots. Gelatin sepharose is suppose to remove fibronectin from the plasma; however, only the high molecular weight fibronectin is removed. The lower molecular weight fragments remain in the solution.

Cell adhesion to recombinant fibrin involves hamster fibronectin, recombinant fibrin, $\alpha 5\beta 1$ and $\alpha v\beta 3$. Both of these integrins recognize fibronectin and $\alpha v\beta 3$ also recognizes fibrin. Again, because there are eight times the number of $\alpha 5\beta 1$ on the cell surface than $\alpha v\beta 3$, blocking the integrin $\alpha 5\beta 1$ will have a huge effect on cell adhesion. There may not be enough $\alpha v\beta 3$ on the surface to sustain cell interaction with the fibrin matrix. However blocking both of these integrins will abolish cell adhesion. This is seen when treating the cells with the disintegrin Kistrin.

In smooth muscle cell contraction of plasma clots, the key players are surface fibronectin, $\alpha 5\beta 1$ and probably to some extent $\alpha v\beta 3$ since blocking both the integrins inhibits contraction greater than blocking either one of them alone. The greater role of surface fibronectin over plasma fibronectin could be due to the nature of the three dimensional assay and the availability of the plasma fibronectin to the cell. Factor XIIIa may crosslink the fibronectin to the fibrin matrix in such a way that it is not easy for the cells to bind to it. Smooth muscle cell contraction of recombinant fibrin clots is probably similar to plasma clot contraction except that hamster fibronectin seems to be more accessible for cell binding due to the lack of Factor XIIIa in the solution.

Conclusion

In conclusion, I have determined that human smooth muscle cells interact with a fibrin matrix through cellular or plasma fibronectin and they

utilize the $\alpha 5\beta 1$ integrin to do so (Figure 6.1). The fact that fibronectin can mediate cell interaction with fibrin is not novel since fibronectin has been shown to be able to bind to fibrin covalently and non covalently^{136, 220}. However, what is novel about this work is that smooth muscle cell interaction with fibrin requires fibronectin. Previous studies on cell interaction with fibrin has shown that $\alpha v\beta 3$ is the direct receptor for fibrin on fibroblasts, endothelial cells, and melanoma cells^{69, 116, 145}. The difference between these cells and human smooth muscle cells is the level of $\alpha v\beta 3$ expression on the cell surface. Human smooth muscle cells express very low levels of $\alpha v\beta 3$ *in vitro* and this low level is also found on intimal smooth muscle cells *in vivo*^{65, 66}. In contrast, expression of $\alpha 5\beta 1$ on human smooth muscle cells is relatively high *in vitro* and *in vivo*. Moreover, smooth muscle cells co-localized with fibrin in atherosclerotic plaques have the same integrin profile with respects to $\alpha v\beta 3$ and $\alpha 5\beta 1$.

The expression of the integrin on the surface of the cell is important when contemplating whether a particular integrin would be a suitable target for pharmaceutical development. As reviewed in Chapter 1, plaque rupture and thrombosis occur during advance stages of atherosclerosis. This can also occur during medical intervention. Angioplasty is a technique used to forcibly reopen and enlarge the lumen of a vessel that has been closed due to atherosclerosis. During angioplasty, the vessel is severely wounded and fibrin accumulates in the wall. In this situation, wound healing would play an important part in the maintenance of the vessel wall while wound contraction may explain the incidence of restenosis, a decrease in lumen area. One component of wound healing is smooth muscle cells interaction with fibrin through integrin receptors. If smooth muscle cells can cause pathological narrowing during the healing of the vessel wall, then preventing smooth muscle cell interaction with fibrin would be a way to decrease the incidences of restenosis after angioplasty.

A pharmaceutical reagent that inhibits the integrins $\alpha v\beta 3$ and $\alpha IIb\beta 3$ has already been developed. Reopro is a humanized antibody against the fibrin receptors, $\alpha v\beta 3$ and $\alpha IIb\beta 3$. In clinical trials, Reopro decreased the clinical incidence of restenosis significantly and this protection has lasted over two years^{114, 148, 176}. It was hypothesized that the effectiveness of Reopro was due to its ability to block both platelet and smooth muscle cell

interaction with ligands of $\alpha v \beta 3$ and $\alpha IIb \beta 3$ found in the atherosclerotic plaque and the vessel wall. However, work cited in this dissertation has demonstrated that intimal smooth muscle cells do not express the integrin $\alpha v \beta 3$; therefore, Reopro must not be effective in preventing smooth muscle interaction with ligands of $\alpha v \beta 3$, including fibrin and fibronectin. Smooth muscle cell migration and proliferation are important components of atherosclerosis progression and a reagent that could prevent these events would be an effective drug in inhibiting atherosclerosis and restenosis.

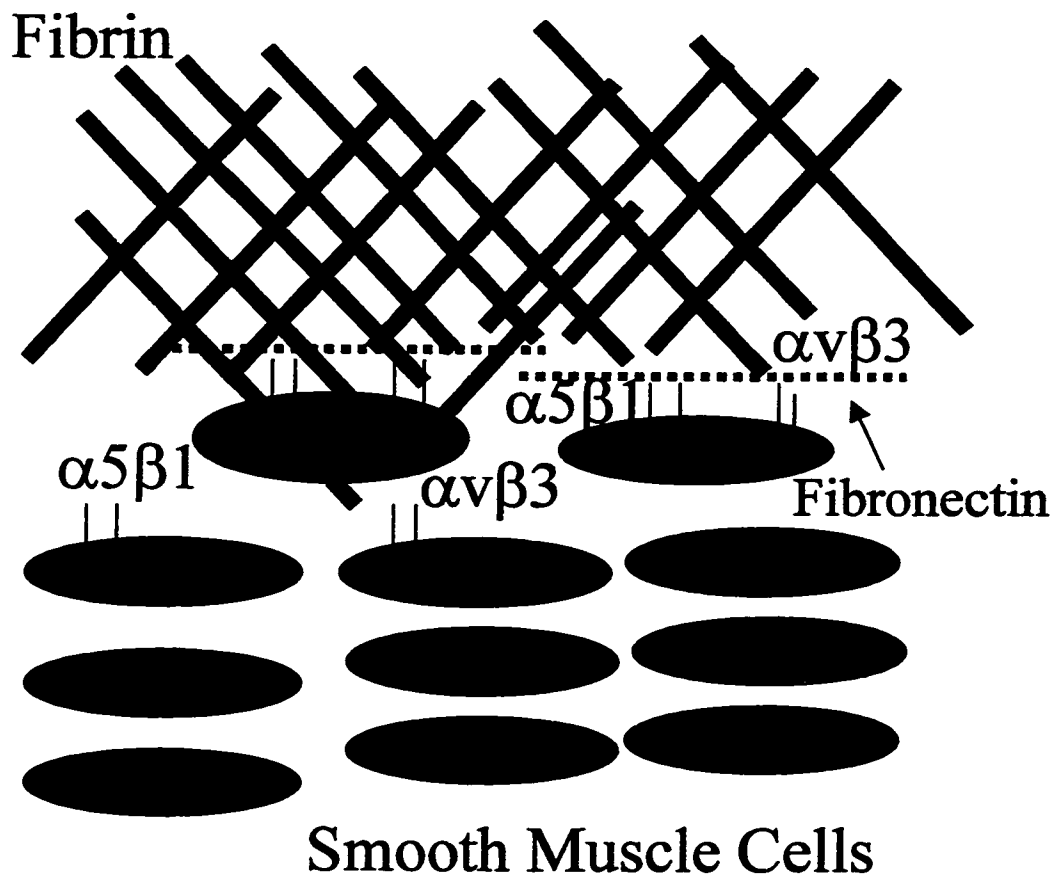


Figure 6.1: Model of Smooth Muscle Cell Interaction with Fibrin

Future Directions

The initial characterization of human smooth muscle cell interaction with fibrin has been presented in this dissertation. The knowledge gained will aid other researchers in their search for pharmaceutical targets in the prevention of atherosclerosis progression and restenosis. Even though a number of key components in smooth muscle cell interaction with fibrin have been identified, a number of new questions have been raised.

The first area of further research is the role of fibronectin in smooth muscle cell interaction with fibrin. Small molecular fragments of fibronectin have been identified in all three sources of fibrinogen and the role of these fibronectin fragments is unknown in regards to smooth muscle cell-fibrin interaction. Are these fragments causing fibronectin to polymerize? Are they inhibiting fibronectin assembly on the cell surface? Are cells interacting with these fragments? Do the concentrations of these fragments confer different experimental results between plasma clots and recombinant fibrin? To address these questions, the identification of some of the fragments through peptide sequencing should be carried out. In particular, the presence of the III₁-C fragment in the sources of fibrinogen should be confirmed. To determine if the smaller molecular weight fibronectins are important in smooth muscle cell interaction with fibrin, fibronectin can be cleaved using a number of proteases. These smaller molecular weight fragments can be mixed with the recombinant fibrinogen and the effect on smooth muscle cell adhesion and contraction of fibrin clots can be assessed.

The second area of research may be the role of the integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ on smooth muscle cell interaction with fibronectin and fibrin. When we initiated this project, we assumed that the integrins would be receptors for the extracellular matrices. However, in the course of our studies, we realized that integrins can assemble the fibronectin matrix on the cell surface and can also transduce signals to the cell nucleus. The studies presented here utilize many anti-integrin antibodies and peptides. It would be interesting to assess the affect of these inhibitors on fibronectin secretion, fibronectin matrix assembly and expression of other proteins on the cell

surface. There has also been much work presented in the literature on crosstalk between the integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ ¹⁶⁹. In smooth muscle cell interaction with fibrin, anti- $\alpha 5\beta 1$ antibodies inhibited cell-matrix interactions and by blocking both the $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins, the extent of inhibition increased. I hypothesized that this was partly due to the different amounts of $\alpha v\beta 3$ and $\alpha 5\beta 1$ on the cell surface. However, this result could be due to intracellular signaling between the two integrins.

The third promising area of research is the role of Factor XIIIa and tissue transglutaminase in smooth muscle interaction with fibrin. In the data presented in this dissertation, I have assumed that Factor XIIIa is crosslinking fibronectin to fibrin in the plasma clots. Crosslinking of fibronectin to fibrin should be confirmed through SDS-PAGE analysis. It is not known for instance whether all of the fibronectin fragments are being cross-linked to fibrin. Cross-linking these fragments together may prevent the cells from interacting with the fibronectin in a clot contraction assay. It is not known whether the smooth muscle cells used in these studies are able to secrete tissue transglutaminase. Since many other cell types such as fibroblasts and endothelial cells are able to secrete tissue transglutaminase, I assume that smooth muscle cells are also able to do this. If the cells are able to secrete tissue transglutaminase, then the proteins in the recombinant fibrin clot may be cross-linked together by tissue transglutaminase. Because factor XIIIa and tissue transglutaminase cross link proteins using different bonds, these hypotheses can be tested using Western blot analysis. The role of Factor XIIIa or tissue transglutaminase should be explored in regards to cell spreading and fibrin clot contraction.

A fourth area of further research development would be to characterize the mechanism of action of Kistrin. Kistrin had a huge effect in inhibiting smooth muscle cell interaction to all three different sources of fibrin. These exploratory experiments could be the first steps in determining the potential of Kistrin as a pharmaceutical reagent. The answers to the following questions are still not known and would make a fascinating area of research. Is the molecule actually inhibiting all the possible cell-matrix interactions? Where is Kistrin binding on the cell surface? Is it changing

the affinity state of any of the integrins? Would Kistrin be effective in an animal model of atherosclerosis?

Lastly, it is still not known how smooth muscle cells and fibronectin are interacting with the fibrin molecule. There are two integrin recognition sites on fibrinogen: two RGD sequences and a dodecapeptide sequence. It is not clear in the literature which RGD sites are available for cell binding. It is known that the dodecapeptide site is recognized by the α IIb β 3 integrin^{42, 62, 221, 222}. Initial experiments using antibodies against each of the RGD sites in fibrinogen to inhibit smooth muscle cell-fibrin interaction gave conflicting results. Therefore more work should be carried out to determine how the cells are interacting with fibrin.

Bibliography

1. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature*. 1993; 362:801-809.
2. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ. Compensatory enlargement of human atherosclerotic coronary arteries. *The New England Journal of Medicine*. 1987; 316:1371-1375.
3. Clarkson TB, Prichard RW, Morgan TM, Petrick GS, Klein PS. Remodeling of coronary arteries in human and non human primates. *JAMA*. 1994; 271:289-294.
4. Keren G. Compensatory, enlargement, remodeling and restenosis. *Advances in Experimental Medical Biology*. 1997; 430:187-196.
5. McGill HC. Arteries too good to kill. *JAMA*. 1994; 271:317-318.
6. Rokitanski Cv. A Manuel of Pathological Anatomy. Vol. 4. London: Sydenham Society, 1852:261.
7. Virchow R. Der atheromatous prozess der arteries. *Wien Med Wochenschr*. 1858; 6:825-829.
8. Fuster V, Ip JH, Badimon L, Badimon JJ, Stein B, Chesebro JH. Importance of experimental models for the development of clinical trials on thromboatherosclerosis. *Circulation*. 1991; 83:IV15-IV25.
9. Salunke NV, Topoleski LDT. Biomechanics of atherosclerotic plaque. *Critical Reviews in Biomechical Engineering*. 1997; 25:243-285.
10. Duguid JB. Thrombosis as a factor in the pathogenesis of coronary atherosclerosis. *Journal of Pathology Bacteriology*. 1946; 1946:207-212.
11. Duguid JB. Thrombosis as a factor in the pathogenesis of aortic atherosclerosis. *Journal of Pathology and Bacteriology*. 1948; 60:57-61.
12. Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell. *Science*. 1973; 180:1332-1339.
13. Ross R. The pathogenesis of atherosclerosis: An update. *New England Journal of Medicine*. 1986; 314:488-500.
14. Schachter M. The pathogenesis of atherosclerosis. *International Journal of Cardiology*. 1997; 62:S3-S7.
15. Fuster V. Mechanisms leading to myocardial infarction: insights from studies of vascular biology. *Circulation*. 1994; 90:2126-2146.

16. Ruoslahti E, Engvall E. Integrins and vascular extracellular matrix assembly. *Journal of Clinical Investigation*. 1997; 100:S53-S56.
17. Rubin E. Blood Vessels. Pathology. Philadelphia: J.B. Lippincott Company, 1988:452-495.
18. Majesky MW, Schwartz SM. An origin for smooth muscle cells from endothelium? *Circulation Research*. 1997; 80:601-603.
19. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, William Insull J, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. *Circulation*. 1995; 92:1355-1374.
20. Schachter M. Vascular smooth muscle cell migration, atherosclerosis, and calcium blockers. *International Journal of Cardiology*. 1997; 62:S85-S90.
21. Libby P, Sukhova G, Lee RT, Liao JK. Molecular biology of atherosclerosis. *International Journal of Cardiology*. 1997; 62:S23-S29.
22. Campbell JH, Campbell GR. The cell biology of atherosclerosis-new developments. *Australia New Zealand Journal of Medicine*. 1997; 27:497-500.
23. Grenholdt M-LM, Dalager-Pedersen S, Falk E. Coronary atherosclerosis: determinants of plaque rupture. *European Heart Journal*. 1998; 19:C24-C29.
24. Gordon D, Reidy MA, Benditt EP, Schwartz SM. Cell proliferation in human coronary arteries. *Proc. Natl. Acad. Sci. USA*. 1990; 87:4600-4604.
25. O'Brien ER, Alpers CE, Steward DK, Ferguson M, Tran N, Gordon D, Benditt EP, Hinohard T, Simpson JB, Schwartz SM. Proliferation in primary and restenotic coronary atherectomy tissue: Implications for anti-proliferative therapy. *Circulation Research*. 1993; 73:223-231.
26. Davies MJ, Richardson PD, Woolf N, Katz DR, Mann J. Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content. *British Heart Journal*. 1993; 69:377-381.
27. Falk E, Shah PK, Fuster V. Coronary plaque disruption. *Circulation*. 1995; 92:657-671.
28. Moreno PR, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT. Macrophage infiltration in acute coronary syndromes. *Circulation*. 1994; 90:775-778.

29. Shah PK, Falk E, Badimon JJ, Fernandez-Ortiz A, Maihac A, Villareal-Levy G, Fallon JT, Regnstrom J, Fuster V. Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques. *Circulation*. 1995; 95:1565-1569.
30. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: Basic Mechanisms. *Circulation*. 1995; 91:2488-2496.
31. Nermerson Y. Tissue factor: then and now. *Thrombosis and Haemostasis*. 1995; 74:180-184.
32. Grinnell F. Mini-Review on the cellular mechanisms of disease. *Journal of Cell Biology*. 1994; 124:401-404.
33. Martin P. Wound healing-aiming for perfect skin regeneration. *Science*. 1997; 276:75-81.
34. McClain SA, Simon M, Jones E, Handi A, Gailit JO, Tonnesen MG, Newman D, Clark RAF. Mesenchymal cell activation is the rate-limiting step of granulation tissue induction. *American Journal of Pathology*. 1996; 149:1257-1270.
35. Tranquillo RT, Murray JD. Mechanistic model of wound contraction. *Surgical Research*. 1993; 55:233-247.
36. Rudolph R, Berg JV, Ehrlich HP. Wound contraction and scar contracture. In: Cohen IK, Diegelmann RF, Lindblad WJ, eds. *Wound Healing: Biochemical and Clinical Aspects*. Vol. 96. Philadelphia: W.B. Saunders, 1992:114.
37. Courtman DW, Schwartz SM, Hart CE. Sequential injury of the rabbit abdominal aorta induces intramural coagulation and luminal narrowing independent of intimal mass: extrinsic pathway inhibition inhibits luminal narrowing. *Circulation Research*. 1998; 82:996-1006.
38. Gabbiani B, Hirschel BJ, Ryan GB, Statkov PR, Majno G. Granulation tissue as a contractile organ: a study of structure and function. *Journal of Experimental Medicine*. 1972; 135:719-733.
39. Geary RL, Nikkari ST, Wagner WD, Williams JK, Adams MR, Dean RH. Wound healing: a paradigm for lumen narrowing after arterial reconstruction. *Journal of Vascular Surgery*. 1998; 27:96-108.

40. Colman RW, Hirsh J, Marder VJ, Salzman EW. Fibrinogen structure and physiology. Hemostasis and Thrombosis. Philadelphia: J.B. Lippincott Company, 1994:277-300.
41. Weisel JW, Francis CW, Nagaswami C, Marder VJ. Determination of the topology of factor XIIIa-induced fibrin γ -chain cross-links by electron microscopy of ligated fragments. *Journal of Biological Chemistry*. 1993; 268:26618-26624.
42. Kloczewiak M, Timmons S, Hawiger J. Localization of a site interacting with human platelet receptor on carboxy-terminal segment of human fibrinogen γ chain. *Biochemical and Biophysical Research Communication*. 1982; 107:181-188.
43. Kloczewiak M, Timmons S, Lukas TJ, Hawiger J. Platelet receptor recognition site on human fibrinogen. synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the γ chain. *Biochemistry*. 1984; 23:1767-1774.
44. Shainoff JR, Page IH. Deposition of modified fibrinogen within the aortic intima. *Atherosclerosis*. 1972; 16:287-305.
45. Smith EB, Alexander KM, Massie IB. Quantitative studies on the relationship between insoluble 'fibrin', soluble fibrinogen and low density lipoprotein. *Atherosclerosis*. 1976; 23:19-25.
46. Shekhonin BV, Tararak EM, Samokhin GP, Mitkevich OV, Mazurov AV, Vinogradov DV, Vlasik TN, Kalantarov GF, Koteliansky VE. Visualization of apoB, fibrinogen/fibrin, and fibronectin in the intima of normal and human aorta and large arteries and during atherosclerosis. *Atherosclerosis*. 1990; 82:213-226.
47. Bini A, Kudryk BJ. Fibrinogen and fibrin in the arterial wall. *Thrombosis Research*. 1994; 75:337-341.
48. Valenzuela R, Shainoff JR, DiBello PM, Urbanic DA, Anderson JM, Matsueda GR, Kudryk BJ. Immunoelectrophoretic and immunohistochemical characterizations of fibrinogen derivatives in atherosclerotic aortic intimas and vascular prosthesis pseudo-intimas. *American Journal of Pathology*. 1992; 141:861-880.
49. Haust MD, Wylie JC, More RH. Electron Microscopy of fibrin in human atherosclerotic lesions. *Experimental and Molecular Pathology*. 1965; 4:205-216.

50. Smith EB. Fibrin deposition and fibrin degradation products in atherosclerotic plaques. *Thrombosis Research*. 1994; 75:329-335.
51. Lowe GDO. Fibrinogen and cardiovascular disease: historical introduction. *European Heart Journal*. 1995; 16:2-5.
52. Stirk CM, Kochhar A, Smith EB, Thompson WD. Presence of growth-stimulating fibrin degradation products containing fragment E in human atherosclerotic plaques. *Atherosclerosis*. 1993; 103:159-169.
53. Smith EB. Fibrinogen, fibrin and the arterial wall. *European Heart Journal*. 1995; 16:11-15.
54. Kao VCY, Wissler RW. A study of the immunohistochemical localization of serum lipoproteins and other plasma proteins in human atherosclerotic lesions. *Experimental and Molecular Pathology*. 1965; 4:465-479.
55. Romanic AM, Arleth AJ, Willette RN, Ohlstein EH. Factor XIIIa cross-links lipoprotein(a) with fibrinogen and is present in human atherosclerotic lesions. *Circulation Research*. 1998; 83:264-269.
56. Sahni A, Odrlic T, Francis CW. Binding of basic fibroblast growth factor to fibrinogen and fibrin. *Journal of Biological Chemistry*. 1998; 273:7554-7559.
57. Albelda SM, Buck CA. Integrins and other cell adhesion molecules. *FASEB*. 1990; 4:2868-2880.
58. Haas TA, Plow EF. Integrin-ligand interactions: a year in review. *Current Opinion in Cell Biology*. 1994; 6:656-662.
59. Charo IF, Nannizzi L, Phillips DR, Hsu MA, Scarborough RM. Inhibition of fibrinogen binding to gp IIb-IIIa by a gp IIIa peptide. *Journal of Biological Chemistry*. 1991; 266:1415-1421.
60. D'Souza SE, Ginsberg MH, Burke TA, Lam SCT, Plow EF. Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science*. 1988; 242:91-93.
61. D'Souza SE, Ginsberg MH, Burke TA, Plow EF. The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its α subunit. *Journal of Biological Chemistry*. 1990; 265:3440-3446.

62. D'Souza SE, Ginsberg MH, Matsueda GR, Plow EF. A discrete sequence in a platelet integrin is involved in ligand recognition. *Nature*. 1991; 350:66-68.
63. Bacon-Baguley T, Kudryk BJ, Walz DA. Thrombospondin interaction with fibrinogen. *Journal of Biological Chemistry*. 1987; 262:1927-1930.
64. Skinner MP, Raines EW, Ross R. Dynamic expression of $\alpha 1\beta 1$ and $\alpha 1\beta 2$ integrin receptors by human vascular smooth muscle cells. *American Journal of Pathology*. 1994; 145:1070-1081.
65. Hoshiga M, Alpers CE, Smith LL, Giachelli CM, Schwartz SM. $\alpha v\beta 3$ Integrin expression in normal and atherosclerotic artery. *Circulation Research*. 1995; 77:1129-1135.
66. Ikari Y, Hatsukami TS, Alpers CE, Yee KO, Giachelli CM, Schwartz SM. Alpha V beta 3 integrin is rarely expressed on migrating smooth muscle cells into fibrin matrix in carotid arteries[abstract]. *Circulation*. 1997; 96 (Supplement I):I-667.
67. Ylanne J, Chen Y, O'Toole TE, Loftus JC, Takada Y, Ginsberg MH. Distinct functions of integrin α and β subunit cytoplasmic domains in cell spreading and formation of focal adhesions. *Journal of Cell Biology*. 1993; 122:223-233.
68. Chen Y-P, O'Toole TE, Leong L, Liu B-Q, Diaz-Gonzalez F, Ginsberg MH. $\beta 3$ integrin-mediated fibrin clot retraction by nucleated cells: differing behavior of $\alpha IIb\beta 3$ and $\alpha v\beta 3$. *Blood*. 1995; 86:2606-2615.
69. Katagiri Y, Hiroyama T, Akamatsu N, Suzuki H, Yamazaki H, Tanoue K. Involvement of $\alpha v\beta 3$ integrin in mediating fibrin gel retraction. *Journal of Biological Chemistry*. 1995; 270:1785-1790.
70. Schiro JA, Chan BM, Roswit WT, Kassner PD, Pentland AP, Hemler ME, Eisen AZ, Kupper TS. Integrin alpha 2 beta 1 (VLA-2) mediates reorganization and contraction of collagen matrices by human cells. *Cell*. 1991; 67:403-410.
71. Schwartz SM, Campbell GR, Campbell JH. Replication of smooth muscle cells in vascular disease. *Circulation Research*. 1986; 58:427-444.
72. Fager G, Hansson GK, Gown AM, Larson DM, Skalli O, Bondjers G. Human arterial smooth muscle cells in culture: inverse relationship between proliferation and expression of contractile proteins. *In Vitro Cellular and Developmental Biology*. 1989; 25:511-520.

73. Kakuta T, Currier JW, Horten K, Faxon DP. Failure of compensatory enlargement, not neointimal formation, accounts for lumen narrowing after angioplasty in the atherosclerotic rabbit. *Circulation*. 1993; 88:I -619 (Abstr.).
74. Weissman NJ, Mendelsohn FO, Palacios IF, Weyman AE. Development of coronary compensatory enlargement in vivo: sequential assessments with intravascular ultrasound. *American Heart Journal*. 1995; 130:1283-1285.
75. Davies MJ. Acute coronary thrombosis-the role of plaque disruption and its initiation and prevention. *European Heart Journal*. 1995; 17:3-7.
76. Raghow R. The role of extracellular matrix in postinflammatory wound healing and fibrosis. *The FASEB Journal*. 1994; 8:823-831.
77. Ruosolathi E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. *Science*. 1987; 238:491-497.
78. Wright SD, Weitz JI, Huang AJ, Levin SM, Silverstein SC, Loike JD. Complement receptor type three (CD11b/Cd18) of human polymorphonuclear leukocytes recognizes fibrinogen. *Proceedings National Academy of Science*. 1988; 85:7734-7738.
79. Peerschke EI. The platelet fibrinogen receptor. *Seminars in Hematology*. 1985; 22:241-249.
80. Altieri DC, Bader R, Mannucci PM, Edgington TS. Oligospecificity of the cellular adhesion receptor Mac-1 encompasses an inducible recognition for fibrinogen. *Journal of Cell Biology*. 1988; 107:1893-1900.
81. Marshall Jf, Rutherford DC, McCartney ACE, Mitjans F, Goodman SL, Hart IR. $\alpha v \beta 1$ is a receptor for vitronectin and fibrinogen, and acts with $\alpha 5 \beta 1$ to mediate spreading on fibronectin. *Journal of Cell Science*. 1995; 108:1227-1238.
82. Neugebauer KM, Venstrom KA, Reichardt LF. Adhesion of a chicken myeloblast cell line to fibrinogen and vitronectin through a $\beta 1$ -class integrin. *Journal of Cell Biology*. 1992; 116:809-815.
83. Suehiro K, Gailit J, Plow EF. Fibrinogen is a ligand for integrin $\alpha 5 \beta 1$ on endothelial cells. *Journal of Biological Chemistry*. 1997; 272:5360-5366.
84. Barbieri B, Balconi G, Dejana E, Donati MB. Evidence that vascular endothelial cells can induce the retraction of fibrin clots (41260). *Proceedings of the Society for Experimental Biology and Medicine*. 1981; 168:204-207.

85. Cheresh DA, Spiro RC. Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen and von Willebrand factor. *Journal of Biological Chemistry*. 1987; 262:17703-17711.
86. Wayner EA, Pardo AG, Humphries MJ, McDonald JA, Carter WG. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (cs-1) in plasma fibronectin. *Journal of Cell Biology*. 1989; 109:1321-1330.
87. de-Vries JE, Keizer GD, te-Velde AA, Voordouw A, Ruiter D, Rümke P, Spits H, Figdor CG. Characterization of melanoma-associated surface antigens involved in the adhesion and motility of human melanoma cells. *International Journal of Cancer*. 1986; 38:465-473.
88. Caixia S, Stewart S, Wayner E, Carter W, Wilkins J. Antibodies to different members of the $\beta 1$ (CD29) integrins induce homotypic and heterotypic cellular aggregation. *Cellular Immunology*. 1991; 138:216-228.
89. Giancotti FG, Ruoslahti E. Elevated levels of the $\alpha 5\beta 1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. *Cell*. 1990; 60:849-859.
90. Carter WG, Wayner EA, Bouchard TS, Kaur P. The role of integrins $\alpha 2\beta 1$ and $\alpha 3\beta 1$ in cell-cell and cell-substrate adhesion of human epidermal Cells. *Journal of Cell Biology*. 1990; 110:1387-1404.
91. Wayner EA, Carter WG, Piotrowicz RS, Kunicki TJ. The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix; preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. *Journal of Cell Biology*. 1988; 107:1881-1891.
92. Wayner EA, Orlando RA, Cheresh DA. Integrins alpha v beta 3 and alpha v beta 5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *Journal of Cell Biology*. 1991; 113:919-929.
93. Wayner EA, Carter WG. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique alpha and common beta subunits. *Journal of Cell Biology*. 1987; 105:1873-1884.

94. Pierschbacher MD, Ruoslahti E. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. *Journal Of Biological Chemistry*. 1987; 262:17294-17298.
95. Pierschbacher MD, Hayman EG, Ruoslahti E. Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell*. 1981; 26:259-267.
96. Akiyama SK, Yamada SS, Chen WT, Yamada KM. Analysis of fibronectin receptor function with monoclonal antibodies: Roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *Journal of Cell Biology*. 1989; 109:863-875.
97. Akiyama SK, Larjava H, Yamada KM. Differences in the biosynthesis and localization of the fibronectin receptor in normal and transformed cultured human cells. *Cancer Research*. 1990; 50:1601-1607.
98. Collier BS. A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb/IIIa complex. *Journal of Clinical Investigation*. 1985; 76:101-108.
99. Collier BS, Scudder LR. Inhibition of dog platelet function by *in vivo* infusion of F(ab')₂ fragments of a monoclonal antibody to the platelet glycoprotein IIb/IIIa receptor. *Blood*. 1985; 66:1456-1459.
100. Investigators TE. Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. *The New England Journal of Medicine*. 1994; 330:956-961.
101. Arroyo AG, S'anchez-Mateos P, Campanero MR, Mart'in-Padura I, Dejana E, S'anchez-Madrid F. Regulation of the VLA integrin-ligand interactions through the beta 1 subunit. *Journal of Cell Biology*. 1992; 117:659-670.
102. Bergelson JM, Chan BM, Finberg RW, Hemler ME. The integrin VLA-2 binds echovirus 1 and extracellular matrix ligands by different mechanisms. *Journal of Clinical Investigation*. 1993; 92:232-.
103. Wang A, Yokosaki Y, Ferrando R, Balmes J, Sheppard D. Differential regulation of airway epithelial integrins by growth factors. *American Journal of Respiratory Cell and Molecular Biology*. 1996; 15:664-672.
104. Damsky CH, Fitzgerald ML, Fisher SJ. Distribution patterns of extracellular matrix components and adhesion receptors are intricately

- modulated during first trimester cytotrophoblast differentiation along the invasive pathway, *in vivo*. *Journal of Clinical Investigation*. 1992; 89:210-222.
105. Preissner KT, Holzhueter S, Justus C, Muller-Berghaus G. Identification and partial characterization of platelet vitronectin: evidence for complex formation with platelet-derived plasminogen activator inhibitor-1. *Blood*. 1989; 74:1989-1996.
106. Real FX, Houghton AN, Abino AN, Cardo CC, Melamed MR, Oettgen HF, Old LJ. Surface antigens on melanomas and melanocytes defined by mouse monoclonal antibodies: specificity analysis and comparisons of antigen expression in cultured cells and tissues. *Cancer Research*. 1985; 45:4401-4411.
107. Liaw L, Skinner MP, Raines EW, Ross R, Cheresch DA, Schwartz SM, Giachelli CM. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. *Journal of Clinical Investigation*. 1995; 95:713-724.
108. Perez-Reyes N, Halbert CL, Smith PP, Benditt EP, McDougall JK. Immortalization of primary human smooth muscle cells. *Proc. Natl. Acad. Sci. USA*. 1992; 89:1224-1228.
109. Engvall E, Ruoslahti E. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *International Journal of Cancer*. 1977; 20:1-5.
110. Vuento M, Vaheri A. Purification of fibronectin by affinity chromatography. *Biochemical Journal*. 1979; 183:331-337.
111. Gorkun OV, Veklich YI, Weisel JW, Lord ST. The conversion of fibrinogen to fibrin: recombinant fibrinogen typifies plasma fibrinogen. *Blood*. 1997; 89:4407-4414.
112. Rooney MM, Parise LV, Lord ST. Dissecting clot retraction and platelet aggregation. *Journal of Biological Chemistry*. 1996; 271:8553-8555.
113. Liaw L, Almeda M, Hart CE, Schwartz SM, Giachelli CM. Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells *in vitro*. *Circulation Research*. 1994; 74:214-224.
114. Topol EJ. Prevention of cardiovascular ischemic complications with new platelet glycoprotein IIb/IIIa inhibitors. *American Heart Journal*. 1995; 130:666-672.

115. Giltay JC, Mourik JAv. Structure and function of endothelial cell integrins. *Haemostasis*. 1988; 18:376-389.
116. Smith RA, Mosesson MW, Rooney MM, Lord ST, Daniels AV, Gartner TK. The role of putative fibrinogen A-alpha, B-beta and gamma-A chain integrin binding sites in endothelial cell mediated clot contraction. *Journal Biological Chemistry*. 1997; 272:22080-22085.
117. Belkin VM, Belkin AM, Koteliansky VE. Human smooth muscle VLA-1 integrin: purification, substrate specificity, localization in aorta, and expression during development. *Journal of Cell Biology*. 1990; 111:2159-2170.
118. Gotwals PJ, Chi-Rosso G, Lindner V, Yang J, Ling L, fawell SE, Koteliansky VE. The $\alpha 1\beta 1$ Integrin is expressed during neointima formation in rat arteries and mediates collagen matrix reorganization. *Journal of Clinical Investigation*. 1996; 97:1-9.
119. Cremona O, Savoia P, Marchisio PC, Gabbiani B, Chapponier C. The $\alpha 6$ and $\beta 4$ integrin subunits are expressed by smooth muscle cells of human small vessels: a new localization in mesenchymal cells. *Journal of Histochemistry and Cytochemistry*. 1994; 42:1221-1228.
120. Mechtersheimer G, Barth T, Quentmeier A, Moller P. Differential expression of $\beta 1$ integrins in nonneoplastic smooth and striated muscle cells and in tumors derived from these cells. *American Journal of Pathology*. 1994; 144:1172-1182.
121. Clyman RI, MacDonald KA, Kramer RH. Integrin receptors on aortic smooth muscle cells mediate adhesion to fibronectin, laminin and collagen. *Circulation Research*. 1990; 67:175-186.
122. Xu J, Clark RAF. Extracellular matrix alters PDGF regulation of fibroblast integrins. *The Journal of Cell Biology*. 1996; 132:239-249.
123. Naito M, Hayashi T, Kuzuya M, Funaki C, Asai K, Kuzuya F. Effects of fibrinogen and fibrin on the migration of vascular smooth muscle cells in vitro. *Atherosclerosis*. 1990; 83:9-14.
124. Naito M, Funaki C, Hayashi T, Yamada K, Asai K, Yoshimine N, Kuzuya F. Substrate-bound fibrinogen, fibrin and other cell attachment-promoting proteins as a scaffold for cultured vascular smooth muscle cells. *Atherosclerosis*. 1992; 96:227-234.

125. Yee KO, Rooney MM, Giachelli CM, Lord ST, Schwartz SM. Role of $\beta 1$ and $\beta 3$ integrins in human smooth muscle cell adhesion to and contraction of fibrin clots *in vitro*. *Circulation Research*. 1998; 83:241-251.
126. Tamkun JW, Hynes RO. Plasma fibronectin is synthesized and secreted by hepatocytes. *Journal of Biological Sciences*. 1983; 258:4641-4647.
127. Hynes RO, Yamada KM. Fibronectins: multifunctional modular glycoproteins. *Journal of Cell Biology*. 1982; 95:369-377.
128. Hynes R. Molecular biology of fibronectin. *Annual Review of Cellular Biology*. 1985; 1:67-90.
129. Matsuka YV, Medved LV, Brew SA, Ingham KC. The NH₂-terminal fibrin-binding site of fibronectin is formed by interacting fourth and fifth finger domains. *Journal of Biological Sciences*. 1994; 269:9539-9546.
130. Atherton BT, Hynes RO. A difference between plasma and cellular fibronectins located with monoclonal antibodies. *Cell*. 1981; 25:133-141.
131. Pearlstein E, Gold LI, Garcia-Pardo A. Fibronectin: A review of its structure and biological activity. *Molecular and Cellular Biochemistry*. 1980; 29:103-128.
132. Jones GE, Arumugham RG, Tanzer ML. Fibronectin glycosylation modulates fibroblast adhesion and spreading. *Journal of Cell Biology*. 1986; 103:1663-1670.
133. Charo IF, Nannizzi L, Smith JW, Cheresch DA. The vitronectin receptor $\alpha v \beta 3$ binds fibronectin and acts in concert with $\alpha 5 \beta 1$ in promoting cellular attachment and spreading on fibronectin. *Journal of Cell Biology*. 1990; 111:2795-2800.
134. Cheng Y-F, Clyman RI, Enenstein J, Waleh N, Pytela R, Kramer RH. The integrin complex $\alpha v \beta 3$ participates in the adhesion of microvascular endothelial cells to fibronectin. *Experimental Cell Research*. 1991; 194:69-77.
135. Mosesson MW, Amrani DL. The structure and biologic activities of plasma fibronectin. *Blood*. 1980; 56:145-158.
136. Okada M, Blombäck B, Chang M-D, Horowitz B. Fibronectin and fibrin gel structure. *Journal of Biological Chemistry*. 1985; 260:1811-1820.
137. Stathakis NE, Mossesson MW. Interaction among heparin, cold-insoluble globulin, and fibrinogen in formation of the heparin precipitable fraction of plasma. *Journal of Clinical Investigation*. 1977; 60:855-865.

138. Stathakis NE, Mossesson MW, Chen AB, Galanakis DK. Cryoprecipitation of fibrin-fibrinogen complexes induced by cold-insoluble globulin of plasma. *Blood*. 1978; 51:1211-1222.
139. Sekiguchi K, Hakomori S. Domain structure of human plasma fibronectin. Differences and similarities between human and hamster fibronectins. *Journal of Biological Chemistry*. 1983; 258:3967-3973.
140. Grinnell F, Feld M, Minter D. Fibroblast adhesion to fibrinogen and fibrin substrata: requirement for cold-insoluble globulin (plasma fibronectin). *Cell*. 1980; 19:517-525.
141. Weiss E, Yamaguchi Y, Falabella A, Crane S, Tokuda Y, Falanga V. Un-cross-linked fibrin substrates inhibit keratinocyte spreading and replication: correction with fibronectin and factor XIII cross-linking. *Journal of Cellular Physiology*. 1998; 174:58-65.
142. Carter WG. The cooperative role of the transformation-sensitive glycoproteins, GP140 and fibronectin, in cell attachment and spreading. *Journal of Biological Chemistry*. 1982; 257:3249-3257.
143. Colanzi A, Mironov A, Weigert R, Limina C, Flati S, Cericola C, Tullio GD, Girolamo MD, Corda D, Matteis MAD, Luini A. Brefeldin A-induced ADP-ribosylation in the structure and function of the golgi complex. *Advances in Experimental Medicine and Biology*. 1997; 419:331-335.
144. Sciaky N, Presley J, Smith C, Zaal KJM, Cole N, Moreira JE, Terasaki M, Siggia E, Lippincott-Schwartz J. Golgi tubule traffic and the effects of Brefeldin A visualized in living cells. *Journal of Cell Biology*. 1997; 139:1137-1155.
145. Niewiarowski S, Regoeczi E, Mustard JF. Adhesion of fibroblasts to polymerizing fibrin and retraction of fibrin induced by fibroblasts. *Proc. Soc. Exp. Biol. Med.* 1972; 140:199-204.
146. Glagov S. Intimal hyperplasia, vascular modeling, and the restenosis problem. *Circulation*. 1994; 89:2888-2891.
147. Koenig W. Recent progress in the clinical aspects of fibrinogen. *European Heart Journal*. 1995; 16:54-59.
148. Topol EJ, Ferguson JJ, Weisman HF, Tchong JE, Ellis SG, Kleiman NS, Ivanhoe RJ, Wang AL, Miller DP, Anderson KM, Califf RM. Long term protection from myocardial ischemic events in a randomized trial of brief

- integrin beta 3 blockade with percutaneous coronary intervention. EPIC Investigation Group. Evaluation of platelet IIb/IIIa inhibition for prevention of ischemic complications. *JAMA*. 1997; 278:479-484.
149. Le-Breton H, Plow EF, Topol EJ. Role of platelets in restenosis after percutaneous coronary revascularization. *Journal of the American College of Cardiology*. 1996; 28:1643-1651.
150. Matsuno H, Stassen JM, Vermylen J, Deckmyn H. Inhibition of integrin function by a cyclic RGD-containing peptide prevents neointima formation. *Circulation*. 1994; 90:2203-2206.
151. Matsuno H, Stassen JM, Moons L, Vermylen J, Hoylaerts MF. Neointima formation in injured hamster carotid artery is effectively prevented by the combination G4120 and quinapril. *Thrombosis and Haemostasis*. 1996; 76:263-269.
152. Choi ET, Engel L, Callow AD, Sun S, Trachtenberg J, Santoro S, Ryan US. Inhibition of neointimal hyperplasia by blocking $\alpha\beta 3$ integrin with a small peptide antagonist GpenGRGDSPCA. *Journal of Vascular Surgery*. 1994; 19:125-134.
153. Leavesley DI, Schwartz MA, Rosenfeld M, Cheresch DA. Integrin $\beta 1$ and $\beta 3$ mediated endothelial cell migration is triggered through distinct signaling mechanisms. *Journal Of Cell Biology*. 1993; 121:163-170.
154. Brown SL, Lundgren CH, Nordt T, Fjuii S. Stimulation of migration of human aortic smooth muscle cells by vitronectin: implications for atherosclerosis. *Cardiovascular Research*. 1994; 28:1815-1820.
155. Woodard AS, Garcia-Cardena G, Leong M, Madri JA, Sessa WC, Languino LR. The synergistic activity of $\alpha\beta 3$ integrin and PDGF receptor increases cell migration. *Journal of Cell Science*. 1998; 111:469-478.
156. Yee KO, Rooney MM, Lord ST, Schwartz SM. Cell surface fibronectin is the ligand responsible for integrin mediated fibrin gel contraction. *in preparation*. 1998.
157. Lusinskas FW, Lawler J. Integrins as dynamic regulators of vascular function. *The FASEB Journal*. 1994; 8:929-938.
158. Barker PL, Bullens S, Bunting S, Burdick DJ, Chan KS, Deisher T, Eigenbrot C, Gadek TR, Gantz R, Lipari MT, Muir CD, Napier MA, Pitti RM, Padua A, Quan C, Stanley M, Struble M, Tom JYK, Burnier JP. Cyclic

- RGD peptide analogues as antiplatelet antithrombotics. *Journal of Medicinal Chemistry*. 1992; 35:2040-2048.
159. Jackson DY, Quan C, Artis DR, Rawson T, Blackburn B, Struble M, Fitzgerald G, Chan k, Mullins S, Burnier JP, Fairbrother WJ, Clark K, Berisini M, Chui H, Renz M, Jones S, Fong S. Potent $\alpha 4\beta 1$ peptide antagonists as potential anti-inflammatory agents. *Journal of Medicinal Chemistry*. 1997; 40:3359-3368.
160. Dennis MS, Henzel WJ, Pitti RM, Lipari MT, Napier MA, Deisher TA, Bunting S, Lazarus RA. Platelet glycoprotein IIb-IIIa protein antagonists from snake venoms: evidence for a family of platelet-aggregation inhibitors. *Proceedings of the National Academy of Science*. 1990; 87:2471-2475.
161. Chuntharapai A, Bodary S, Horton M, Kim KJ. Blocking monoclonal antibodies to alpha v beta 3 integrins: a unique epitope of alpha v beta 3 integrin is present on human osteoclasts. *Experimental Cell Research*. 1993; 205:345-352.
162. Tselepis VH, Green LJ, Humphries MJ. An RGD to LDV motif conversion within the disintegrin kistrin generates an integrin antagonist that retains potency but exhibits altered receptor specificity. *Journal of Biological Chemistry*. 1997; 272:21341-21348.
163. Mogford JE, Davis GE, Platts SH, Meininger GA. Vascular smooth muscle $\alpha v\beta 3$ integrin mediates arteriolar vasodilation in response to RGD peptides. *Circulation Research*. 1996; 79:821-826.
164. Jones JI, Prevetie T, Gockerman A, Clemmons DR. Ligand occupancy of the $\alpha v\beta 3$ integrin is necessary for smooth muscle cells to migrate in response to insulin-like growth factor 1. *Proceedings of the National Academy of Science*. 1996; 93:2482-2487.
165. Lu X, Deadman JJ, Williams JA, Kakkar VV, Rahman S. Synthetic RGD peptides derived from the adhesive domains of snake-venom proteins: evaluation as inhibitors of platelet aggregation. *Biochemical Journal*. 1993; 296:21-24.
166. Carderelli PM, Cobb RR, Nowlin DM, Scholz W, Gorcsan F, Moscinski M, Yasuhara M, Chiang S-L, Lobl TJ. Cyclic RGD peptide inhibits $\alpha 4\beta 1$ interaction with connecting segment 1 and vascular cell adhesion molecule. *Journal of Biological Chemistry*. 1994; 269:18668-18673.

167. Pacifici R, Roman J, Kimble R, Civitelli R, Brownfield CM, Bizzarri C. Ligand binding to monocyte $\alpha 5\beta 1$ integrin activates the $\alpha 2\beta 1$ receptor via the $\alpha 5$ cytoplasmic domain and protein kinase C. *Journal of Immunology*. 1994; 153:2222-2233.
168. Blystone SD, Graham IL, Lindberg FP, Brown EJ. Integrin $\alpha v\beta 3$ differentially regulates adhesive and phagocytic functions of the fibronectin receptor $\alpha 5\beta 1$. *Journal of Cell Biology*. 1994; 127:1129-1137.
169. Blystone SD, Lindberg FP, LaFlamme SE, Brown EJ. Integrin $\beta 3$ cytoplasmic tail is necessary and sufficient for the regulation of $\alpha 5\beta 1$ phagocytosis by $\alpha v\beta 3$ and integrin-associated protein. *Journal of Cell Biology*. 1995; 130:745-754.
170. Adler M, Lazarus R, Dennis MS, Wagner G. Solution structure of kistrin, a potent platelet aggregation inhibitor and GPIIb-IIIa antagonist. *Science*. 1991; 253:445-448.
171. Rucinski B, Niewiarowski S, Holt JC, Soszka T, Knudsen KA. Batroxostatin, an Arg-Gly-Asp-containing peptide from *Bothrops atrox*, is a potent inhibitor of platelet aggregation and cell interaction with fibronectin. *Biochimica et Biophysica Acta*. 1990; 1054:257-262.
172. Yasuda T, Gold HK, Leinbach RC, Yaoita H, Fallon JT, Guerrero L, Napier MA, Bunting S, Collen D. Kistrin, a polypeptide platelet GPIIb/IIIa receptor antagonist, enhances and sustains coronary arterial thrombolysis with recombinant tissue-type plasminogen activator in a canine preparation. *Circulation*. 1991; 83:1038-1047.
173. Topol EJ, Califf RM, Weisman HF, Ellis SG, Tcheng JE, Worley S, Ivanhoe R, George BS, Fintel D, Weston M, et al. Randomised trial of coronary intervention with antibody against platelet IIb/IIIa integrin for reduction of clinical restenosis: results at six months. The EPIC Investigators. *Lancet*. 1994; 343:881-886.
174. Aguirre FV, Topol EJ, Ferguson JJ, Anderson K, Blankenship JC, Heuser RR, Sigmon K, Taylor M, Gottlieb R, Hanovich G, et al. Bleeding complications with the chimeric antibody to platelet glycoprotein IIb/IIIa integrin in patients undergoing percutaneous coronary intervention. EPIC Investigators. *Circulation*. 1995; 91:2882-2890.
175. Lincoff AM, Califf RM, Anderson KM, Weisman HF, Aguirre FV, Kleiman NS, Harrington RA, Topol EJ. Evidence for prevention of death

- and myocardial infarction with platelet membrane glycoprotein IIb/IIIa receptor blockade by abciximad (c7E3 Fab) among patients with unstable angina undergoing percutaneous coronary revascularization. EPIC Investigators. Evaluation of 7E3 in Preventing Ischemic Complications. *Journal of American College of Cardiology*. 1997;30:149-156.
176. Moliterno DJ, Califf RM, Aguirre FV, Anderson K, Sigmon KN, Weisman HF, Topol EJ. Effect of platelet glycoprotein IIb/IIIa integrin blockade on activated clotting time during percutaneous transluminal coronary angioplasty or directional atherectomy (the EPIC trial). Evaluation of c7E3 Fab in the Prevention of Ischemic Complications trial. *American Journal of Cardiology*. 1995;75:559-562.
177. Tcheng JE, Ellis SG, Beorge BS, Kereiakes DJ, Kleiman NS, Talley JD, Wang AL, Weisman HF, Califf RM, Topol EJ. Pharmacodynamics of chimeric glycoprotein IIb/IIIa integrin antiplatelet antibody Fab 7E3 in high-risk coronary angioplasty. *Circulation*. 1994;90:1757-1764.
178. Tcheng JE. Glycoprotein IIb/IIIa receptor inhibitors: putting the EPIC, IMPACT II, RESTORE, and EPILOG trials in perspective. *American Journal of Cardiology*. 1996;78:35-40.
179. Collier BS, Anderson K, Weisman HF. New antiplatelet agents: platelet GPIIb/IIIa antagonists. *Thrombosis and Haemostasis*. 1995;74:302-308.
180. Felding-Habermann B, Ruggeri ZM, Cheresch DA. Distinct biological consequences of integrin $\alpha v\beta 3$ mediated melanoma cell adhesion to fibrinogen and its plasmic fragments. *Journal of Biological Chemistry*. 1992;267:5070-5077.
181. Bosmans JM, Kockx MM, Vrints CJ, Bult H, Meyer GRYD, Herman AG. Fibrin(ogen) and von willebrand factor deposition are associated with intimal thickening after balloon angioplasty of the rabbit carotid artery. *Arteriosclerosis Thrombosis Vascular Biology*. 1997;17:634-645.
182. Montalescot G, Ankri A, Vicaut E, Drobinski G, Grosgeat Y, Thomas D. Fibrinogen after coronary angioplasty as a risk factor for restenosis. *Circulation*. 1995;92:31-38.
183. Naito M, Nomura H, Iguchi A. Migration of cultured vascular smooth muscle cells into non-crosslinked fibrin gels. *Thrombosis Research*. 1996;84:129-136.

184. Bini A, Fenoglio JJ, Mesa-Tejada R, Kudryk B, Kaplan KL. Identification and distribution of fibrinogen, fibrin and fibrin(ogen) degradation products in atherosclerosis. *Arteriosclerosis*. 1989; 9:109-121.
185. Guadiz G, Sporn LA, Simpson-Haidaris PJ. Thrombin cleavage-independent deposition of fibrinogen in extracellular matrices. *Blood*. 1997; 90:2644-2653.
186. Odrlijin TM, Francis CW, Sporn LA, Bunce LA, Marder VJ, Simpson-Haidaris PJ. Heparin-binding domain of fibrin mediates its binding to endothelial cells. *Arteriosclerosis Thrombosis Vascular Biology*. 1996; 16:1544-1551.
187. Bini A, Callender S, Procyk R, Blömbäck B, Kudryk BJ. Flow and antibody binding properties of hydrated fibrins prepared from plasma, platelet rich plasma and whole blood. *Thrombosis Research*. 1994; 76:145-156.
188. Courtney MA, Bunce LA, Neroni LA, Simpson-Haidaris PJ. Cloning of the complete coding sequence of rat fibrinogen B beta chain cDNA: interspecies conservation of fibrin beta 15-42 primary structure. *Blood Coagulation Fibrinolysis*. 1994; 5:487-496.
189. Hamaguchi M, Bunce LA, Sporn LA, Francis CW. Plasminic degradation of fibrin decreases platelet adhesion and spreading. *Blood*. 1994; 84:1143-1150.
190. Zacharski LR, Memoli VA, Ornstein DL, Rousseau SM, Kisiel W, Kudryk BJ. Tumor cell procoagulant and urokinase expression in carcinoma of the ovary. *Journal of National Cancer Institute*. 1993; 85:1225-1230.
191. Procyk R, Kudryk B, Callender S, Blömbäck B. Accessibility of epitopes on fibrin clots and fibrinogen gels. *Blood*. 1991; 77:1469-1475.
192. Kannel WB, Wolf PA, Castelli WP, D'Agostino RB. Fibrinogen and risk of cardiovascular disease. The Framingham Study. *Jama*. 1987; 258:1183-1186.
193. Wilhelmsen L, Svardsudd K, Kcrsan-Bengtson K, Larsson B, Welin L, Tibblin G. Fibrinogen as a risk factor for stroke and myocardial infarction. *New England Journal of Medicine*. 1984; 311:501-505.
194. Meade TW, North WR, Chakrabarti R, Stirling Y, Haines AP, Thompson SG, Brozovi'e M. Haemostatic function and cardiovascular death: early results of a prospective study. *Lancet*. 1980; 1:1050-1054.

195. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *American Journal of Pathology*. 1995;146:1029-1039.
196. Gailit J, Clarke C, Newman D, Tonnesen MG, Mosesson MW, Clark RAF. Human fibroblasts bind directly to fibrinogen at RGD sites through Integrin $\alpha v \beta 3$. *Experimental Cell Research*. 1997;232:118-126.
197. Tanoue K, Katagiri Y, Suzuki H. Molecular mechanisms of fibrin gel clot contraction by platelets and cultured tumor cells. *Polish Journal of Pharmacology*. 1996;48:341-343.
198. Schafer BM, Maier K, Eickhoff U, Todd RF, Kramer MD. Plasminogen activation in healing human wounds. *American Journal of Pathology*. 1994;144:1269-1280.
199. Clark RAF, Tonnesen MG, Gailit J, Cheresch DA. Transient functional expression of $\alpha v \beta 3$ on vascular cells during wound repair. *American Journal of Pathology*. 1996;148:1407-1421.
200. Henke CA, Roongta U, Mickelson DJ, Knutson JR, McCarthy JB. CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix. *Journal of Clinical Investigation*. 1996;97:2541-2552.
201. Svec K, White J, Vaillant P, Jessurun J, Roongta U, Krumwiede M, Johnson D, Henke C. Acute lung injury fibroblast migration and invasion of a fibrin matrix is mediated by CD44. *Journal of Clinical Investigation*. 1996;98:1713-1727.
202. Loike JD, Silverstein R, Wright SD, Weitz JI, Huang AJ, Silverstein SC. The role of protected extracellular compartments in interaction between leukocytes, and platelets, and fibrin/fibrinogen matrices. *Annals of the New York Academy of Science*. 1992;667:163-172.
203. Nachman RL, Leung LL, Kloczewiak M, Hawiger J. Complex formation of platelet membrane glycoproteins IIb and IIIa with the fibrinogen D domain. *Journal of Biological Chemistry*. 1984;259:8584-8588.
204. Greiling D, Clark RAF. Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix. *Journal of Cell Science*. 1997;110:861-870.

205. Clyman RI, Boetzman BW, Chen YQ, Mauray F, Kramer RH, Pytela R, Schnapp LM. Changes in endothelial cell and smooth muscle cell integrin expression during closure of the ductus arteriosus: an immunohistochemical comparison of the fetal, preterm newborn, and full-term newborn rhesus monkey ductus. *Pediatric Research*. 1996; 40:198-208.
206. Ruggiero F, Comte J, Cabanas C, Garrone R. Structural requirements for alpha 1 beta 1 and alpha 2 beta 1 integrin mediated cell adhesion to collagen. *Journal of Cell Science*. 1996; 109:1865-1874.
207. Slepian MJ, Massia SP, Dehdashti B, Fritz A, Whitesell L. β 3-integrins rather than β 1-integrins dominate integrin-matrix interaction involved in postinjury smooth muscle cell migration. *Circulation*. 1998; 97:1818-1827.
208. Bilato C, Curto KA, Monticone RE, Pauly RR, White AJ, Crow MT. The inhibition of vascular smooth muscle cell migration by peptide and antibody antagonists of the α v β 3 integrin complex is reversed by activated calcium/calmodulin-dependent protein kinase II. *Journal of Clinical Investigation*. 1997; 100:693-704.
209. Ichikawa K, Nakagawa K, Hirano K, Sueishi K. The localization of tissue factor and apolipoprotein(a) in atherosclerotic lesions of the human aorta and their relation to fibrinogen-fibrin transition. *Pathology Research Practice*. 1996; 192:224-232.
210. Marmur JD, Thiruvikraman SV, Fyfe BS, Guha A, Sharma SK, Ambrose JA, Fallon JT, Nemerson Y, Taubman MB. Identification of active tissue factor in human coronary atheroma. *Circulation*. 1996; 94:1226-1232.
211. Thiruvikraman SV, Guha A, Roboz J, Taubman MB, Nemerson Y, Fallon JT. In situ localization of tissue factor in human atherosclerotic plaques by binding of digoxigenin-labeled factors VIIa and X. *Lab Investigation*. 1996; 75:451-461.
212. Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature*. 1998; 394:200-203.
213. Magnusson MK, Mosher DF. Fibronectin: Structure, Assembly and Cardiovascular Implications. *Arteriosclerosis Thrombosis Vascular Biology*. 1998; 18:1363-1370.
214. McDonald JA, Quade BJ, Broekelmann TJ, LaChance R, Forsman K, Hasegawa E, Akiyama S. Fibronectin's cell-adhesive domain and an amino-

- terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. *Journal of Biological Chemistry*. 1987; 262:2957-2967.
215. Ingham KC, Brew SA, Huff S, Litvinovich SV. Cryptic self-association sites in type III modules of fibronectin. *Journal of Biological Chemistry*. 1997; 272:1718-1724.
216. Mercurius KO, Morla AO. Inhibition of vascular smooth muscle cell growth by inhibition of fibronectin matrix assembly. *Circulation Research*. 1998; 82:548-556.
217. Wennerberg K, Lohikangas L, Gullberg D, Pfaff M, Johansson S, Fässler R. $\beta 1$ integrin-dependent and -independent polymerization of fibronectin. *Journal of Cell Biology*. 1996; 132:227-238.
218. Fogerty FJ, Akiyama SK, Yamada K, Mosher DF. Inhibition of binding of fibronectin to matrix assembly sites by anti-integrin ($\alpha 5\beta 1$) antibodies. *Journal of Cell Biology*. 1990; 111:699-708.
219. Yang J, Rayburn H, Hynes RO. Embryonic mesodermal defects in $\alpha 5$ integrin-deficient mice. *Development*. 1993; 119:1093-1105.
220. Corbett SA, Lee L, Wilson CL, Schwarzbauer JE. Covalent cross-linking of fibronectin to fibrin is required for maximal cell adhesion to a fibronectin-fibrin matrix. *Journal of Biological Chemistry*. 1997; 272:24999-25005.
221. Tranqui L, Andrieux A, Hudry-Clergeon G, Ryckewaert J-J, Soyez S, Chapel A, Ginsberg MH, Plow EF, Marguerie G. Differential structural requirements for fibrinogen binding to platelets and to endothelial cells. *Journal of Cell Biology*. 1989; 108:2519-2527.
222. Cheresh DA, Berliner SA, Vicente V, Ruggeri ZM. Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. *Cell*. 1989; 58:945-953.

VITA

Karen O Yee

Education

- 1998 Ph.D. Molecular and Cellular Biology
University of Washington/FHCRC
Seattle, WA
Stephen M. Schwartz, dissertation advisor
- 1991 BA. Biological Sciences with Honors
Dartmouth College, Hanover, NH
Valerie A. Galton, thesis advisor

Professional Societies

- American Society for Cell Biology, 1995-present
Association for Women in Science, Seattle Area Chapter,
1994-present
-Co-chair of the Scholarship Committee 1995-1997;
committee member 1997-1998
-Treasurer 1997-1998
-Web page manager and email distributor 1997-1998
American Association of University Women, 1997-present

Research Experience

- 1990: Research Assistant, Laboratory of Dr. Robert Gross,
Dartmouth College, Hanover, NH
- 1990-1991: Research Assistant, Laboratory of Dr. Valerie Galton,
Dartmouth Medical School, Lebanon, NH
- 1991-1993: Research Assistant, Laboratory of Dr. Leonard Zon,
Children's Hospital, Boston, MA
- 1993 (Fall): Rotation Student, Laboratory of Dr. Robert Eisenman,
FHCRC, Seattle, WA
- 1994 (Win): Rotation Student, Laboratory of Dr. David Kimelman,
University of Washington, Seattle, WA
- 1994 (Spr): Rotation Student, Laboratory of Dr. Dina Mandoli,
University of Washington, Seattle, WA
- 1994-1998 Pre-doctoral Student, Laboratory of Dr. Stephen
Schwartz, University of Washington, Seattle, WA

Teaching Responsibilities

Undergraduate Teaching Assistant-Biology 16: Genetics (Dartmouth College, Spring Quarter, 1990) .
 Undergraduate Teaching Assistant-Biology 15: Cell Biology (Dartmouth College, Winter Quarter, 1991)
 Teaching Assistant-Biochemistry 441: Biochemistry (University of Washington, Winter Quarter, 1995)
 Teaching Assistant-Biochemistry 426: Biochemistry Lab (University of Washington, Spring Quarter, 1995)
 Advisor: Supervised Beverly Neubauer during her summer undergraduate internship in the laboratory of Stephen M. Schwartz

Awards and Honors

1991: Richter Memorial Trust Research Grant
 1991: Senior Honors Thesis: Regulation of Carbamyl Phosphate Synthetase Activity by Thyroid Hormone
 1994-1997: NIH Pre-doctoral Trainee: Molecular and Cellular Biology Training Grant
 1995-1996: Berlex Fellowship
 1996: Finalist in the International Vascular Biology Meeting Poster Competition, Seattle, WA (September, 1996)
 1997-1998 American Association of University Women Fellowship
 1998 Moderator for the 1998 Women in Science and Engineering Conference, Women Are WISE; January 24, 1998 (Seattle WA)
 1998: Keystone Symposia Travel Award- Angiogenesis and Vascular Remodeling Meeting (April, 1998)
 1998: NIH Pre-doctoral Trainee: Cardiovascular Training Grant

Publications

Original Reports:

1. Galton VA, Morganelli CM, Schneider MJ, & Yee K. The role of thyroid hormone in the regulation of hepatic carbamyl phosphate synthetase activity in *Rana catesbeiana*. *Endocrinology*, 129(5): 2298-2304, 1991.
2. Zon LI, Yamaguchi Y, Yee K, Albee EA, Kimura A, Bennett JC, Orkin SH, & Ackerman SJ. Expression of mRNA for the GATA-binding proteins in human eosinophils and basophils: Potential role in gene transcription. *Blood*, 81(12):3234-3241, 1993.

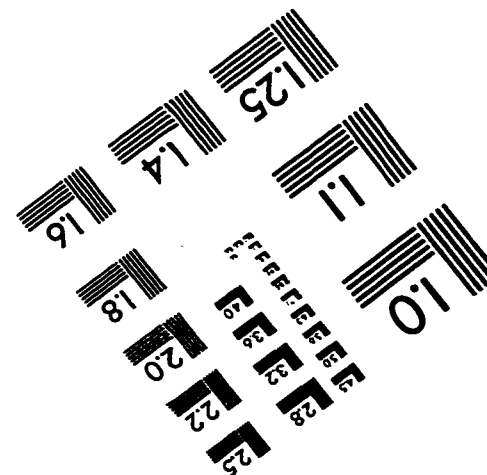
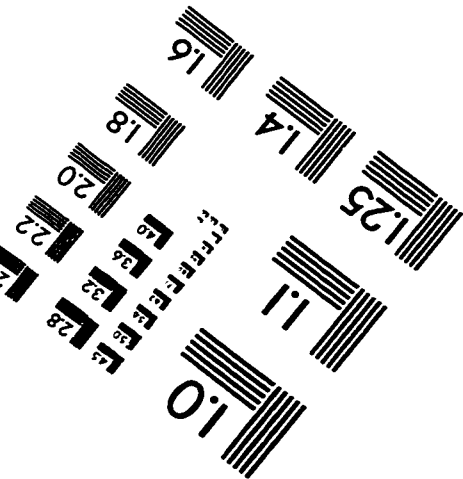
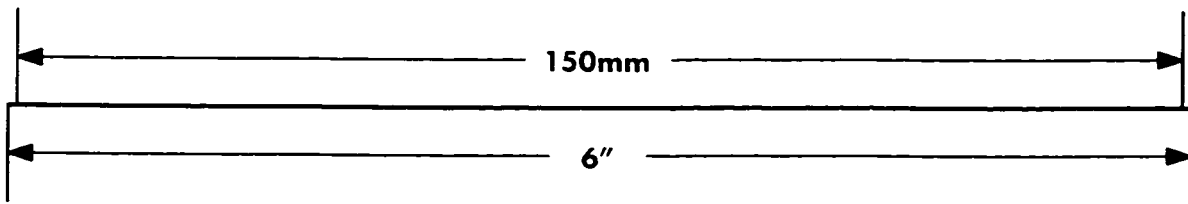
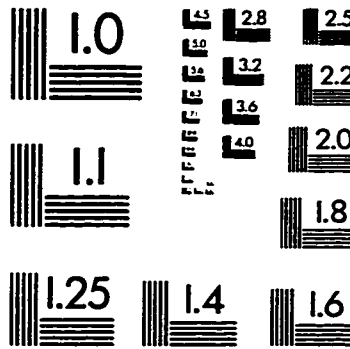
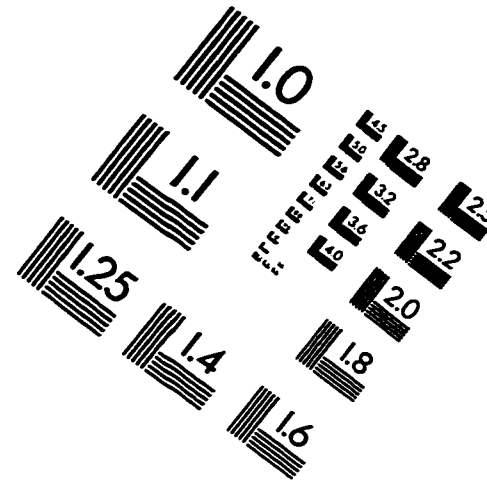
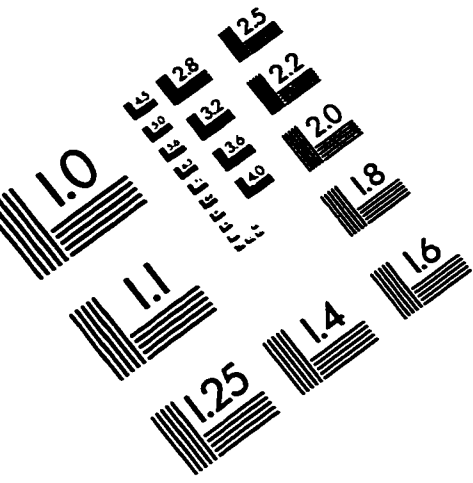
3. Yee K, Bishop TR, Mather C, & Zon LI. Isolation of novel receptor tyrosine kinase cDNA expressed by developing erythroid progenitors. *Blood*, 82(4): 1335-1343, 1993.
4. Yashar BM, Kelley C, Yee K, Errede B, & Zon LI. Novel members of the mitogen-activated protein kinase activator family in *Xenopus laevis*. *Molecular and Cellular Biology*, 13(9): 5738-5748, 1993.
5. Kelley C, Yee K, Harland R, & Zon LI. Ventral expression of GATA-1 and GATA-2 in the *Xenopus* embryo defines induction of hematopoietic mesoderm. *Developmental Biology*, 165(1): 193-205, 1994.
6. Sánchez I, Hughes RT, Mayer BJ, Yee K, Woodgett JR, Avruch J, Kyriakis JM, & Zon LI. Role of SAPK/ERK Kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. *Nature*, 372(6508): 794-798, 1994.
7. Detrich HW-3rd, Kieran MW, Chan FY, Barone LM, Yee K, Rundstadler JA, Pratt S, Ransom D, & Zon LI. Intraembryonic hematopoietic cell migration during vertebrate development. *Proc. Natl. Acad. Sci. USA*, 92(23):10713-10717, 1995.
8. Yee KO, Rooney MM, Giachelli CM, Lord ST, & Schwartz SM. Role of $\beta 1$ and $\beta 3$ integrins in human smooth muscle adhesion to and contraction of fibrin clots. *Circulation Research*, 83(3): 241-251, 1998.
9. Ikari Y, Yee KO, Hatsukami TS, & Schwartz SM. Role of $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins in fibrin dependent smooth muscle cell spreading and migration, and the expression in human carotid arteries. (submitted to *Circulation*)
10. Yee KO, Ikari Y, Bodary S, & Schwartz SM. Kistrin inhibits human smooth muscle cell interaction with fibrin. (submitted to *Circulation Research*).
11. Yee KO, Rooney, MM, Lord ST & Schwartz SM. Cell surface fibronectin is the ligand responsible for integrin mediated fibrin gel contraction. (in preparation).

Abstracts

1. Yee KO Ginsberg MH, Giachelli CM & Schwartz SM. Human aortic smooth muscle cells contract fibrin clots via the $\alpha v\beta 3$ integrin. *Keystone Symposia: Integrins and Signaling Events in Cell Biology and Disease*. Keystone, Colorado, January, 1996.

2. Yee KO, Rooney MM, Ginsberg MH, Giachelli CM Lord ST & Schwartz SM. Human aortic smooth muscle cells contract plasma clots via the $\alpha v \beta 3$ and $\beta 1$ integrins. Gordon Conference: Vascular Cell Biology. Andover, New Hampshire, June, 1996.
3. Yee KO, Rooney MM, Ginsberg MH, Giachelli CM, Lord, ST & Schwartz SM. Human smooth muscle cells contract plasma clots via the $\alpha v \beta 3$ and $\beta 1$ integrins. IX International Vascular Biology Meeting. Seattle, Washington, September, 1996.
4. Yee KO, Rooney MM, Giachelli CM, Lord, ST & Schwartz SM. Human smooth muscle cells contract plasma clots via the $\alpha v \beta 3$ and $\beta 1$ integrins. Vascular Biology Meeting. New Orleans, Louisiana, April, 1997. (Microcirculation)
5. Yee KO, Ikari Y, Rooney MM, Giachelli CM, Lord, ST & Schwartz SM. Human smooth muscle cells interact with fibrin via the $\alpha 5 \beta 1$ integrin. Keystone Symposia: Angiogenesis and Vascular Remodeling. Steamboat Springs, Colorado, April, 1998.

IMAGE EVALUATION TEST TARGET (QA-3)



APPLIED IMAGE, Inc
 1653 East Main Street
 Rochester, NY 14609 USA
 Phone: 716/482-0300
 Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved