EXTRACELLULAR MATRIX-MEDIATED SIGNALING IN THE REGULATION OF VASCULAR SMOOTH MUSCLE CELL PHENOTYPE AND FUNCTION

Joy Roy

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From The Department of Surgical Sciences
Karolinska Institutet
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Joy Roy

Handledare:
Docent Ulf Hedin
Prof Johan Thyberg

Fakultetsopponent:
Professor Andrew C. Newby
Bristol Heart Institute
University of Bristol
Bristol, U.K

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ABSTRACT

Cardiovascular disease is the leading cause of mortality in the world. Even though death rates are today dropping in the developed countries, disability due to the disease is rising worldwide. The major portion of cardiovascular disease associated deaths is due to occlusive atherosclerotic lesions. A major drawback to the surgical treatment of atherosclerosis is the high rate of restenosis or stenosis of bypass grafts that occurs within six months of the procedure. Despite years of research and multiple clinical trials, no effective pharmacological therapy against restenosis or graft stenosis is yet available. This clearly motivates a thorough analysis of the cellular mechanisms involved in these processes. In this thesis, I have focused on the role of the extracellular matrix, and specifically fibronectin (FN), in the regulation of vascular smooth muscle cell (SMC) differentiation and function.

Restenosis is characterized by intimal hyperplasia, which is largely due to SMC migration and proliferation, and deposition of extracellular matrix. SMCs in the normal arterial media, whose primary function is contraction, must first undergo a change in phenotype before they can migrate and proliferate. This modification includes loss of myofilaments and formation of a large endoplasmic reticulum and Golgi complex. SMCs undergo a similar change in phenotype when established in culture either in serum-containing medium or on a substrate of FN under serum-free conditions. Using an in vitro system where freshly isolated rat aortic SMCs are cultured on extracellular matrix components, we have analyzed the intracellular signaling pathways that mediate the effects of FN on SMC phenotype. The results demonstrate that, in addition to the above-mentioned structural reorganization, the transition from a contractile to a synthetic state includes a decrease in caveolae numbers and internalization of caveolin. Caveolae are specialized plasma membrane invaginations with caveolin as the major coat protein and have been implicated in cholesterol transport and signaling. Culture of freshly isolated SMCs on a substrate of FN was further shown to be associated with sustained activation of several signaling molecules, including the small GTP-binding proteins of the Rho family, the extracellular regulated kinases ERK1 and 2, and tyrosine kinases such as focal adhesion kinase (FAK). By using specific inhibitors, we show that activation of integrin-linked tyrosine kinases, ERK1/2 and Rho are necessary for phenotypic modulation. In addition, cyclin D1 was found to be induced as the cells were grown on FN. The induction of this cyclin was also dependent on integrin-linked tyrosine kinase, ERK1/2 and Rho activities. Phenotypic modulation of SMCs in vitro is strongly coupled to cell spreading. Studies in other cell systems have indicated that the Rho proteins regulate the actin cytoskeleton. We show here that this is also the case in SMCs in primary culture and that inhibition of cell spreading using different drugs suppressed the shift in differentiated properties of the cells. Mevinolin, a member of the statin family of lipid-lowering drugs, strongly blocked phenotypic modulation and mitogen-induced DNA synthesis and increased apoptosis. These results suggest that statins, in addition to other described and beneficial effects on the vascular wall, may also function via an effect on SMC phenotypic modulation.

In summary, this thesis has investigated the effects of FN on the differentiated state of SMCs in primary culture. We have demonstrated that caveolae decrease in number concomitant with an internalization of caveolin. This is coupled with activation of signaling molecules such as Rho, ERK1/2 and FAK. In addition, we have targeted cell spreading, an essential process for the shift in phenotype, using a drug that in addition to reducing blood cholesterol also inhibits the activation of Rho proteins. It is our hope that the knowledge gained in studying the cellular signaling mechanisms in control of SMC function will help to develop new strategies to combat restenosis and graft stenosis.

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List of Publications

This thesis is based on the following original articles, referred to in the text by their Roman numerals.

I. Ulf Hedin, Johan Thyberg, Joy Roy, Alexandra Dumitrescu, Phan Kiet Tran. Role of tyrosine kinases in extracellular matrix-mediated modulation of arterial smooth muscle cell phenotype. *Arterioscler Thromb Vasc Biol.* 1997 17:1977-1984

II. Johan Thyberg, Joy Roy, Phan Kiet Tran, Karin Blomgren, Alexandra Dumitrescu, Ulf Hedin. Expression of caveolae on the surface of rat arterial smooth muscle cells is dependent on the phenotypic state of the cells. *Lab Invest* 1997 77: 93-101

III. Joy Roy, Monsur Kazi, Ulf Hedin, Johan Thyberg. Phenotypic modulation of arterial smooth muscle cells is associated with prolonged activation of ERK1/2. *Differentiation* 2001 67: 50-58

IV. Joy Roy, Phan Kiet Tran, Piotr Religa, Monsur Kazi, Karin Lundmark, Ulf Hedin. Fibronectin promotes cell cycle entry in smooth muscle cells in primary culture. *Submitted* 2001

V. Joy Roy, Carl Whatling, Piotr Religa, Ulf Hedin, Johan Thyberg. The HMG-CoA Reductase inhibitor mevinolin blocks phenotypic modulation of rat aortic smooth muscle cells in primary culture. *Manuscript* 2001

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CONTENTS

Supervisor:
Assoc. Professor Ulf Hedin
Department of Surgical Sciences,
Karolinska Institutet,
171 76 Stockholm,
Sweden

Cosupervisor:
Professor Johan Thyberg
Department of Cell and Molecular Biology
Karolinska Instituet
171 77 Stockholm
Sweden

To My Parents

INTRODUCTION

CLINICAL BACKGROUND

The global epidemic of cardiovascular disease

Cardiovascular disease (CVD) has traditionally been seen as the major cause of death in countries of the Western world with a high standard of living. However, 78% of CVD associated deaths in 1999 occurred in the developing world and in 2020, it is predicted that CVD will be the major killer also in developing countries ([Murray and Lopez, 1997]; World Health Report, WHO 2000). Globalization, with changes in socio-economic structure, increased urbanization and industrialization, has brought about lifestyle changes (tobacco use, physical activity, and diet) in these countries and altered population risk factor profiles associated with CVD [Chockalingam, 2000; Murray and Lopez, 1997]. Furthermore, the increasing life expectancy in developing countries due to a decline in infectious and nutritional disorders, prolongs the exposure of the population to CVD risk factors. Even in the Western world, where mortality rates are declining due to improved acute treatment and secondary prevention, CVD is predicted to remain the number one cause of disability in the next 20 years [Murray and Lopez, 1997]. As such, we are facing a global epidemic of CVD (Fig. 1; [Chockalingam, 2000; Fuster, 1999]).

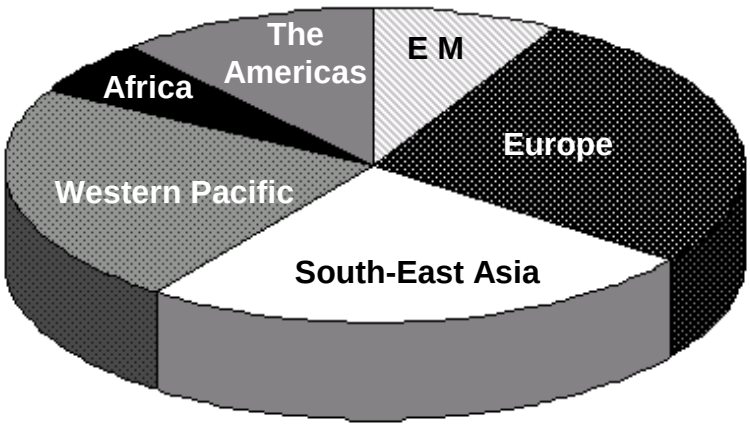

Figure 1: *The numbers of CVD-associated deaths throughout the different regions of the world in 1999 illustrate the ongoing globalization of CVD (EM; Eastern Mediterranean region). Based on the World Health Report, WHO 2000.*

Atherosclerosis, restenosis and vein graft stenosis

The major portion of CVD is caused by atherosclerosis, an inflammatory and fibroproliferative disease in large- and medium-sized arteries [Ross, 1999]. This pathologic process begins as fatty depositions in the artery wall which progress to occlusive and thrombogenic lesions associated with clinical symptoms such as myocardial infarction, stroke and extremity ischemia [Lusis, 2000; Ross, 1999; Stary

et al., 1995]. Cigarette smoking, high blood-cholesterol levels, hypertension, diabetes mellitus, physical inactivity and obesity have been identified as the major risk factors for the disease. Vascular surgery by conventional or endovascular revascularization procedures remains the main treatment for symptomatic occlusive atherosclerotic disease. Today, these procedures consist of a large variety of percutaneous, catheter-based techniques (angioplasty), endarterectomy and by-pass grafting using venous or prosthetic conduits (Fig. 2). The clinical success of the treatment is dependent on the patency of the reconstruction. Within hours to days, thrombus formation may cause early occlusion in the vessel and after a few months up to almost a year after surgery, restenosis and graft stenosis are the major factors that limit long-term patency.

Restenosis is a poorly defined term, referring to the renarrowing of the vessel lumen after vessel injury in association with revascularization procedures. In contrast to restenosis in a diseased artery, graft stenosis takes place in a previously healthy vessel [Hedin and Clowes, 1996]. Over all, about one-third of all patients present with

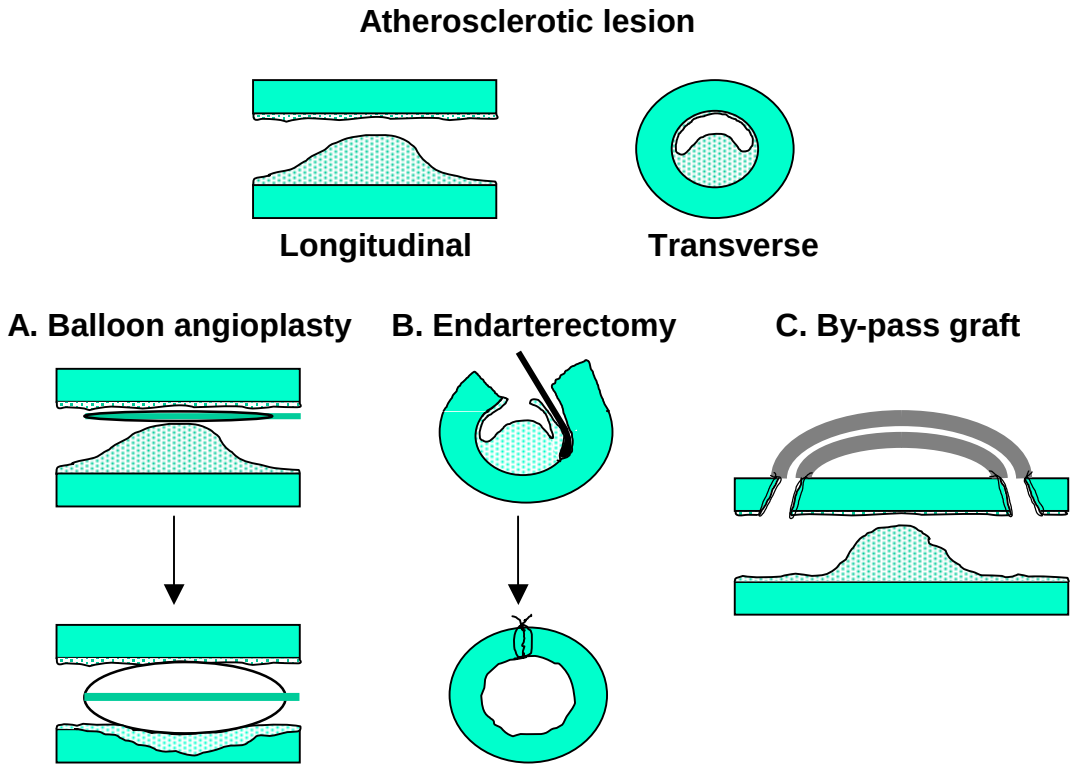

Figure 2: *Surgical treatment for atherosclerosis. A stenotic or occlusive lesion can be treated with balloon angioplasty (A) where a balloon catheter is inserted inside the artery and distended, compressing the plaque into the vessel wall. A plaque can also be removed by endarterectomy (B) where the vessel is opened and the intima with the lesion is excised. Blood flow can be restored in stenosed or occluded vessels by suturing a vein- or synthetic graft (C) to the vessel before and after the diseased segment.*

symptoms of restenosis or vein graft stenosis within 6 months of the procedure [Moliterno and Topol, 1998; Motwani and Topol, 1998]. If one only considers the estimated 1 million percutaneous coronary interventions performed annually worldwide then about 300,000 patients per year will present with symptoms due to restenosis within six months of the procedure [Bittl, 1996; Meyer et al., 1996]. Aside from the well-being of the individual patient, the socio-economic costs are tremendous and growing [Topol et al., 1993]. Most importantly, there is currently no clinically useful approach available to prevent this process.

Initially, restenosis was believed to be due to the proliferation of smooth muscle cells (SMCs) in the intima. Such assumptions were based on results from injury models of normal arteries in laboratory animals without associated vascular disease [Schwartz et al., 1995]. However, further investigations of human restenotic lesions and atherosclerotic animal models have taught us that restenosis is a much more complicated process than first believed [Berk and Harris, 1995; Glagov, 1994]. If one considers the combination of factors that contribute to ultimate narrowing of the vessel lumen after treatment of a stenotic atherosclerotic lesion by balloon angioplasty, restenosis can broadly be divided into four different facets. First, the vessel wall reacts to the acute injury by recoil and the extent of recoil can affect the long term patency of the vessel. Second, the vessel responds to the injury by growing outwards (positive remodeling) or inwards (negative remodeling) affecting the resultant lumen size. Third, acute thrombus formation may subsequently be organized and incorporated into the vessel wall and thereby contribute to the bulk of the intima. Last but not least, intimal hyperplasia due to proliferation and deposition of extracellular matrix (ECM) by SMCs may contribute to luminal narrowing. This process is probably a uniform response to any vessel injury. However, if combined with positive remodeling it can take place without clinically relevant narrowing of the vessel lumen [Moliterno and Topol, 1998]. Figure 3 shows how a combination of these factors may give rise to restenosis. Clinically, a number of factors that predisposes the development of restenosis have been identified such as diabetes mellitus, unstable angina, long lesions, small vessel diameter, and chronic total occlusions. However, the overall predictive value is poor and rarely influences the clinical strategy applied [Bourassa et al., 1991].

In graft stenosis, the injury to the vein during surgery and the hemodynamic changes in the conduit afterwards are the main factors responsible for luminal narrowing [Lemson et al., 2000]. The most obvious similarity between restenosis and vein graft stenosis lies in the activation and proliferation of SMCs and the development of intimal hyperplasia. This process is believed to depend on increased wall tension and altered shear stress forces with or without concomitant mechanical vessel injury and/or endothelial cell loss [Lemson et al., 2000].

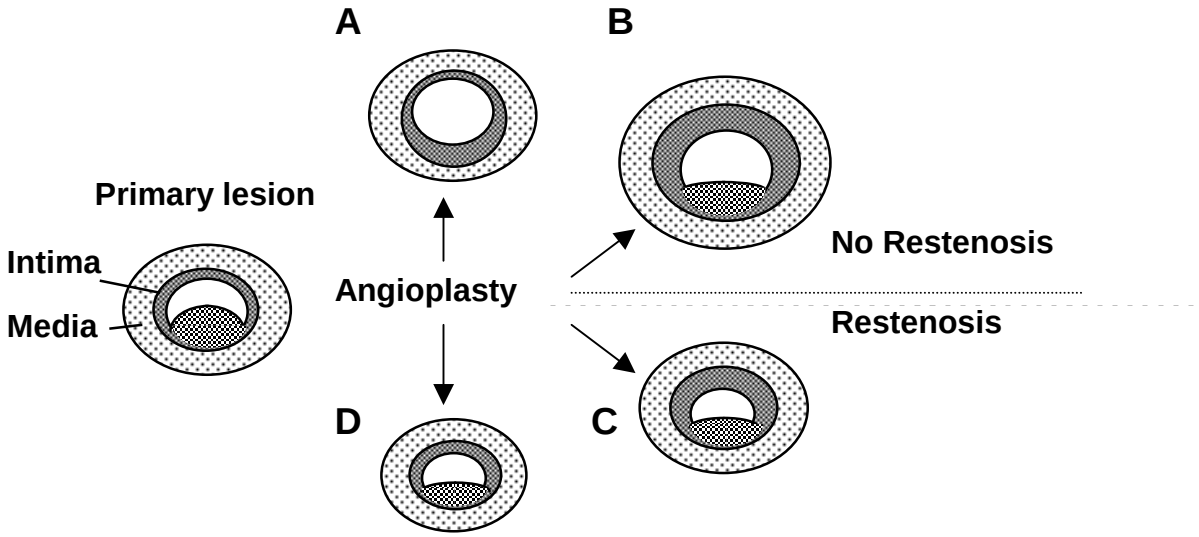

***Figure 3**: Development of restenosis after balloon angioplasty. No restenosis will develop (A) if no intimal hyperplasia is formed and the vessel retains its original diameter or (B) an excessive formation of intimal hyperplasia is counteracted by enlargement of the entire vessel diameter (positive remodeling). Restenosis will develop (C) when excessive intimal hyperplasia is formed in the absence of positive remodeling or (D) by decreased vessel diameter (negative remodeling) in the absence of intimal hyperplasia (adapted from [Hedin, 1997]).*

Management of restenosis and graft stenosis

Numerous clinical trials including thousands of patients have been carried out to test pharmacological therapy mainly against restenosis after coronary angioplasty and to a lesser extent against graft stenosis [Mak and Topol, 1997]. The drugs that have been tested include anti-platelet and anticoagulant agents, lipid-lowering drugs, antioxidants and anti-proliferative agents [Bult, 2000; Mak and Topol, 1997]. In retrospect, and in view of the complexity of the restenotic process, it may not be surprising that drugs targeting one or a few aspects of the disease process have been at best only partly successful [Bult, 2000]. For example, platelet aggregation was targeted using a monoclonal antibody against an adhesion molecule, GPIIb/IIIa located on the surface of platelets and initial clinical studies suggested reduced restenosis rates when this drug was used in association with the revascularisation procedure [EPIC, 1994]. It was proposed that this may have been due to its inhibitory effects on the vitronectin receptor, $\alpha v\beta 3$, and possibly inhibition of SMC migration [Matsuno et al., 1994]. However, further clinical studies have not been able to support this concept [CAPTURE, 1997; EPILOG, 1997; IMPACT-II, 1997; Moliterno and Topol, 1998; RESTORE, 1997]. Statins are lipid-lowering drugs that function through the inhibition of HMG-CoA reductase, an enzyme that catalyzes the rate-limiting step in cholesterol synthesis [Endo et al., 1977]. Clinical trials have been performed using statins to treat restenosis based on the observations that therapeutic serum concentrations of fluvastatin inhibited SMC proliferation in vitro [Corsini et al., 1996;

Serruys et al., 1999]. Although some studies have shown that systemic treatment with statins decrease restenosis rates, larger multicenter studies have not confirmed these findings [Serruys et al., 1999; Weintraub et al., 1994]. Mechanical techniques such as excimer laser ablation and rotational atherectomy have also proved to be unsuccessful, although short term relief for the patient can be achieved by repeated angioplasty [Appelman et al., 1996; Moliterno and Topol, 1998; Reifart et al., 1997]. Another device-based method is stent implantation, where a metallic net (stent) is put into place to prevent the vessel wall from recoil and negative remodeling after balloon dilatation of a stenotic lesion. However, the chronic presence of a foreign body increases thrombogenicity and prolongs vessel wall injury leading to an excessive intimal growth, also known as in-stent restenosis. Despite these drawbacks, stent implantation has been shown to reduce restenosis rates in clinical trials [Fischman et al., 1994; Serruys et al., 1994]. However, the indiscriminate and unnecessary use of stents after balloon angioplasty may be a dangerous development since in-stent restenosis is probably even more difficult to manage than restenosis [Karsch and Newby, 2000; Mach, 2000]. Recently, intravascular irradiation has been applied to overcome restenosis inside stents [Moussavian et al., 2001]. Despite promising preliminary results, the treatment is associated with unwanted side effects such as enhanced intimal hyperplasia at the edges of the treated vessel (so-called 'candy-wrapper effect') and late thrombotic occlusions, probably because of defective healing in the irradiated vessel segment [Virmani and Farb, 1999]. Thus, the final role of brachytherapy in the management of restenosis is far from settled and caution has been raised against the implementation of the method in clinical practice [Sheppard and Eisenberg, 2001].

Where do we stand today?

The globalization of the cardiovascular disease burden coupled with the lack of effective pharmaceuticual therapy against atherosclerosis, with the possible exception of statins, is rapidly increasing the number of patients who will require revascularization interventions. In turn, this will lead to a great increase in the number of patients who present with restenosis and vein graft stenosis. A few years ago, the improved understanding of the pathobiology of restenosis shifted the focus from neointimal hyperplasia to vascular remodeling which generated many clinical trials designed to prevent negative remodeling. The introduction of intravascular stents in clinical practice was indeed associated with reduced restenosis rates [Fischman et al., 1994; Libby and Ganz, 1997; Serruys et al., 1994]. However, the problem with in-stent restenosis has once again shifted the focus to intimal hyperplasia and the role of SMCs in restenosis [Mach, 2000]. In this thesis, I present new information about intracellular signaling pathways involved in the regulation of SMC function in vitro. Hopefully, my results will add to the basic knowledge that is required to develop new

clinically useful treatment strategies in the management of restenosis and vein graft stenosis.

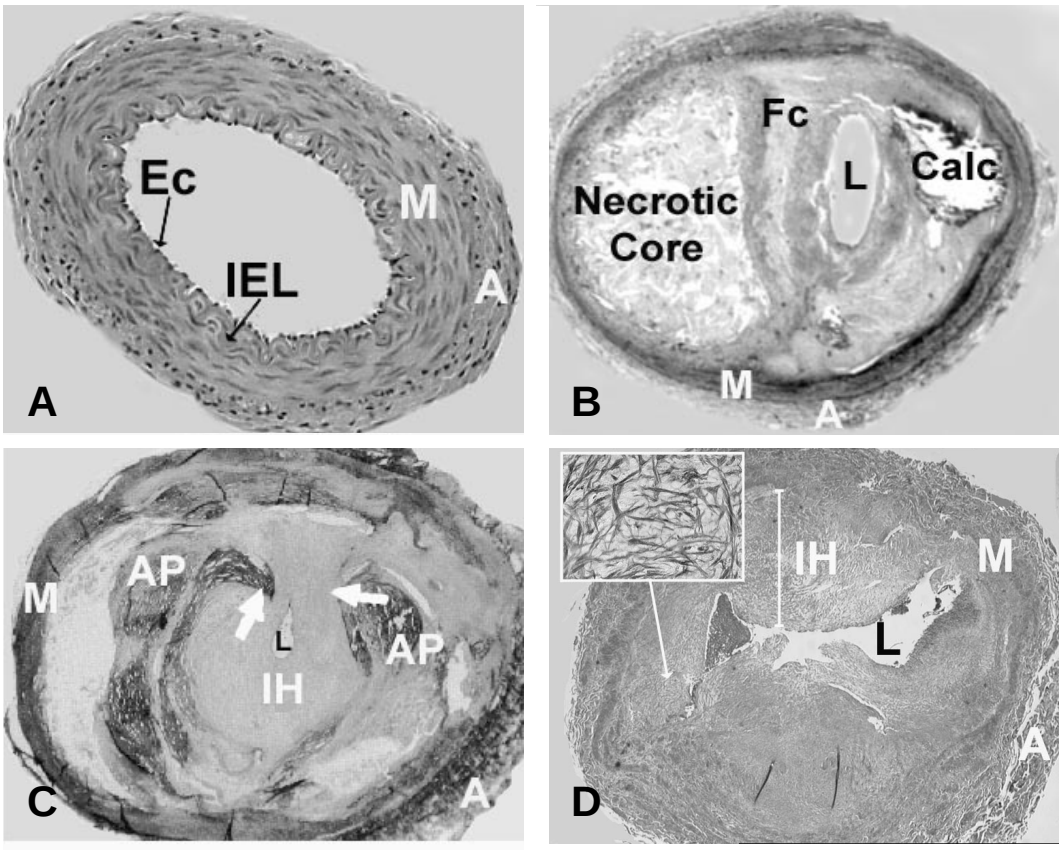

***Figure 4**: Demonstration of the morphology of normal (A) and diseased (B-D) human arteries in transverse sections. (A) Human peripheral artery with the the tunica adventitia (A), tunica media (M), internal elastic lamina (IEL) and endothelial cells (Ec). Note the absence of a marked intima between the IEL and Ec layer. (B) Atherosclerotic lesion with prominent lumen (L) reduction due to the thickened intima with a cholesterol-filled necrotic core, calcification (Calc), and fibrous cap (Fc). (C) Restenotic lesion demonstrating the formation of intimal hyperplasia (IH) after balloon angioplasty in a coronary artery. The original atherosclerotic plaque (AP) which was first treated by angioplasty is seen in the middle of the intima and the tear in the plaque due to the distension of the balloon is marked by arrows. The intimal hyperplasia originating from the media almost completely occludes the lumen of the vessel (modified from [Waller et al., 1991]). (D) Intimal hyperplasia in a vein graft used for hemodialysis. Note the typical appearance of this tissue with stellate-shaped SMCs in a loose ECM (insert). The bar marks the thickness of the intimal hyperplasia.*

CELLULAR BIOLOGY OF THE ARTERY WALL

The normal artery

The arterial wall is divided into 3 major layers, tunica intima, tunica media and tunica adventitia (Fig. 4a). The tunica intima is covered by a monolayer of endothelial cells on top of a basement membrane. The endothelium is more than a semi-permeable barrier to the passage of substances from the blood into the arterial wall. For example,

it has anti-coagulant properties, and is actively involved in the regulation of vascular tone [Kadar and Glasz, 2001]. In humans, the intima has focal thickenings that contain SMCs surrounded by a matrix containing fibronectin (FN), collagen types I, III, V and VI, and the proteoglycans versican and biglycan [Gutierrez et al., 1997; Riessen et al., 1994; Thyberg, 1998a]. The intima is separated from the media by the internal elastic lamina. The tunica media is built up by a network of fenestrated elastic lamellae and circular layers of SMCs, which are the exclusive cellular component in the media. These cells are surrounded by a basement membrane that consists of laminin, collagen type IV, entactin and heparan sulfate proteoglycans such as perlecan [Timpl and Brown, 1996; Wight, 1996]. The interstitial matrix contains collagens type I and III, versican, biglycan and fibronectin [Gutierrez et al., 1997; Riessen et al., 1994]. The external elastic membrane separates the media from the tunica adventitia. The adventitia contains blood vessels (vasa vasorum) and nerves (nervi vascularis) embedded in a collagen-rich connective tissue with fibroblasts and fat cells.

The atherosclerotic plaque

The American Heart Association has classified atherosclerotic lesions into 6 stages based on morphological criteria [Stary et al., 1995]. It begins as adaptive intimal cushions which are composed of SMCs in a proteoglycan-rich matrix. Transmigration of monocytes across the endothelium and uptake of lipoproteins in these cells lead to the formation of fatty streaks, i.e. a subendothelial accumulation of lipid-laden foam cells. The lesions progress and take the form of a lipid core bounded by an endothelialized fibrous cap containing SMCs, macrophages, T-cells and mast cells (Figs. 4b and 5). The advanced lesions also contain calcium deposits and new blood vessels. Atherogenesis involves a complex interaction of lipids, endothelial cells, inflammatory cells, platelets and vascular SMCs [Lusis, 2000; Newby and Zaltsman, 1999; Ross, 1999; Weissberg, 2000]. Among these interactions, subendothelial accumulation of low density lipoproteins (LDL) and its oxidation into oxidized-LDL has a central role [Steinberg, 1997]. This form of LDL is chemotactic for macrophages and stimulates the expression of adhesion molecules on the endothelial cell surface leading to leukocyte infiltration of the intima. Foam cells are formed when macrophages or SMCs take up oxidized-LDL. These various activated cells, together with platelet-derived growth factor (PDGF) released by platelets, act as potent chemoattractants for medial SMCs, which migrate to the intima and proliferate. This process resembles the activation and proliferation of SMCs after arterial injury (discussed below). SMCs break down their basement membrane and produce other matrix components such as fibronectin (FN), collagen type I and elastin. The recruitment of SMCs into the intima facilitates the formation of a fibrous cap, which encages the accumulated foam cells. In the later stages of plaque development, there is a progressive cell death, calcification and an ongoing inflammatory process around the lipid-filled core of the plaque. Death of SMCs, decreased collagen synthesis, and

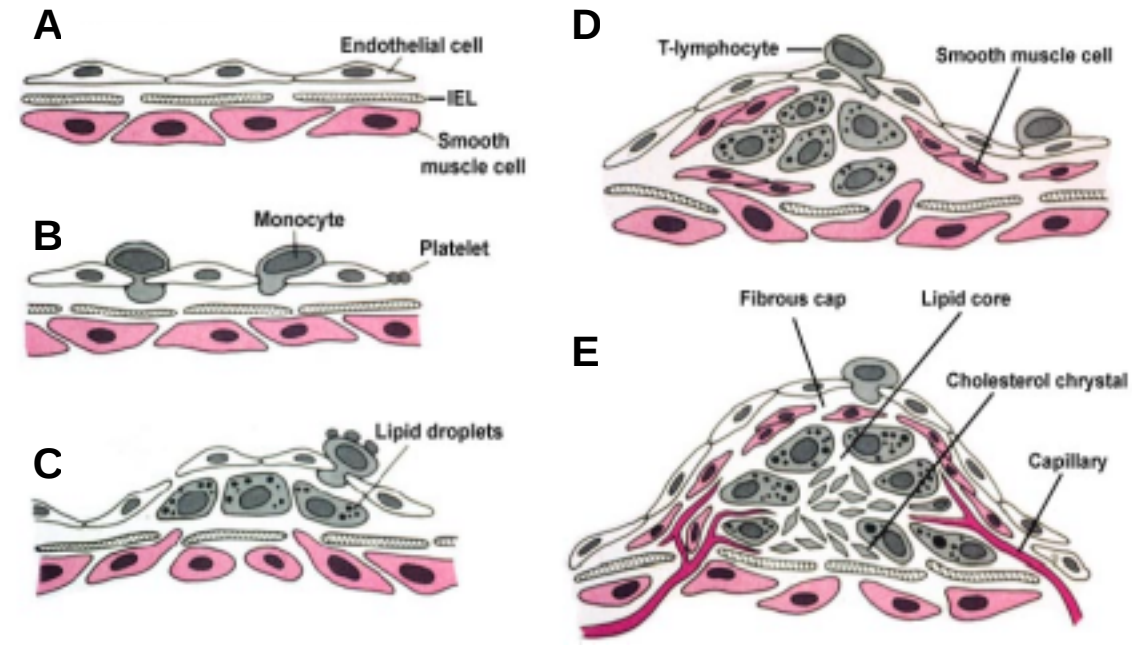

Figure 5: *Schematic description of atherogenesis. (A) Normal arterial wall. In the first phase (B), monocytes adhere to endothelial cells and migrate into the intima where they accumulate lipids and form the fatty streak (C). SMCs are attracted by chemotactic stimuli released from inflammatory cells and by proliferation and secretion of ECM components build up a fibrous cap encaging lipids and lipid-filled foam cells (D). In the advanced lesion, the lipid core is filled with lipids, calcifications, foam cells and necrotic cells, and is surrounded by a fibrous cap made up of SMCs and ECM (E).*

release of matrix metalloproteinases (MMPs) by inflammatory cells finally degrade and weaken the fibrous cap, especially at the edges (shoulder region). This may ultimately lead to plaque rupture, exposure of the thrombogenic lipid core to the blood, thrombosis, vessel occlusion and clinical symptoms [Libby, 2000; Libby et al., 1996]. Thus, the atherosclerotic plaque is complex and the dynamic interaction between the different components determines the clinical outcome [Weissberg, 2000].

Intimal hyperplasia

Intimal hyperplasia is a histological term for the connective tissue formed by cell proliferation and deposition of ECM in the tunica intima of the vessel wall (Figs. 4c and d; [Hedin and Clowes, 1996]). It occurs both physiologically in the ductus arteriosus and in vascular diseases such as atherosclerosis and transplant

arteriosclerosis. The most obvious forms of intimal hyperplasia occur after vessel injury during surgery or trauma (restenosis, vein graft stenosis). Much of our knowledge about intimal hyperplasia comes from animal models, especially from the rat carotid balloon injury model [Libby and Tanaka, 1997; Schwartz et al., 1995]. This model was established by Clowes and coworkers who injured the left common carotid

artery with a small-caliber Fogarty balloon catheter (see Methods; [Clowes et al., 1983a; Clowes et al., 1983b]). Other modes of injury such as ligation or cross clamping of the artery, drying of the endothelium and removal of the adventitia have been shown to give similar results [Schwartz et al., 1995].

The vascular response to balloon injury has been described to occur in three phases [Schwartz et al., 1995]:

Phase 1: Activation of medial SMCs

As early as 24 hours after injury, DNA synthesis has been detected in SMCs in the arterial media [Clowes et al., 1983a]. This is probably due to the release of fibroblast growth factor-2 (also known as basic FGF) bound to heparan sulfate proteoglycans in the ECM and from injured medial cells [Lindner et al., 1991; Lindner and Reidy, 1991]. Analysis of cellular fine structure, using electron microscopy, shows that some of the SMCs in the inner layers have typical synthetic features whereas the rest of the cells retain the contractile features seen in SMCs in uninjured vessels [Thyberg, 1998a; Thyberg et al., 1995]. Conceivably, it is the synthetic SMCs that synthesize DNA in vitro since contractile SMCs need to modulate to a synthetic state before they can enter the cell cycle [Chamley-Campbell and Campbell, 1981; Chamley-Campbell et al., 1981]. The extent of activation of SMCs correlates to the degree of injury. Endothelial denudation activates only the cells in the innermost layers while medial tearing is associated with activation also in the deeper layers of the media [Edelman et al., 1992].

Phase 2: Migration of SMCs

Synthetic SMCs have been detected in the intima within 4-7 days of injury [Thyberg et al., 1995]. It is assumed that the activated SMCs in the media migrate through the internal elastic lamina along a PDGF gradient. PDGF, which is a strong chemotactic factor for SMCs in culture, is released by platelets that aggregate at the surface after injury [Jawien et al., 1992]. This idea is supported by the fact that platelet depletion decreases SMC migration without affecting SMC proliferation [Fingerle et al., 1989].

Phase 3: Intima formation

The first SMCs in the intima are detected about a week after injury. These cells are exclusively in the synthetic state and respond to growth factor stimulation by proliferating and depositing ECM in their vicinity [Thyberg et al., 1995]. The mitogens involved probably include insulin–like growth factor-1, thrombin, angiotensin-II, and endothelin-1 [Thyberg, 1998a]. FGF-2 probably plays a minor role in intimal SMC proliferation since heparin, which binds FGF-2, prevented intima formation when administered immediately but not 6 hours after injury [Lindner et al., 1992]. The intima reaches its maximum size about 2 weeks after injury [Clowes et al., 1983a; Clowes et al., 1983b]. Electron microscopic observations made 5-7 weeks after

injury showed that the neointima retained its size but the SMCs had returned to a contractile state [Thyberg et al., 1995].

The origin of the intimal SMCs has been an issue of debate. The traditional theory [Hayry, 1998; Owens, 1995; Schwartz et al., 1995] that intimal SMCs originate from the media has been challenged by recent studies in animal models of transplant arteriosclerosis and arterial injury, which suggest that intimal SMCs may originate from circulating bone marrow-derived cells [Han et al., 2001; Shimizu et al., 2001]. For example, Han and coworkers used an iliac scratch injury model applied to female mice that had been irradiated and transplanted with bone marrow from male mice. By probing for the Y-chromosome, about 50% of the neointimal cells were seen to be of male origin [Han et al., 2001]. In another mouse study, aorta allograft recipients were transplanted with bone marrow cells that expressed β-galactosidase. This study showed that almost all cells in the intima were of bone marrow origin [Shimizu et al., 2001]. In line with these results, Religa and coworkers from our laboratory have detected SMα-actin expressing cells of bone marrow origin in intimal hyperplasia both after balloon carotid injury and in aortic transplants in rats (personal communication). Another hypothesis is that medial SMCs that migrate and proliferate in the intima possess a specific phenotype [Neuville et al., 1997; Schwartz et al., 1995]. In culture, rat intimal SMCs are epithelioid and grow as a monolayer. SMCs with a similar phenotype have been found in cultures of medial SMCs and therefore suggested to be the origin of intimal SMCs [Orlandi et al., 1994; Walker et al., 1986]. The cellular retinol binding protein-1 (CRBP-1) has been proposed to be a marker of these cells [Neuville et al., 1997]. However, data from our laboratory show that CRBP is induced in SMCs cultured on FN (Fig. 6), suggesting that the phenotype of the SMCs is dependent on the ECM rather than a function of their origin (Roy et al., unpublished data).

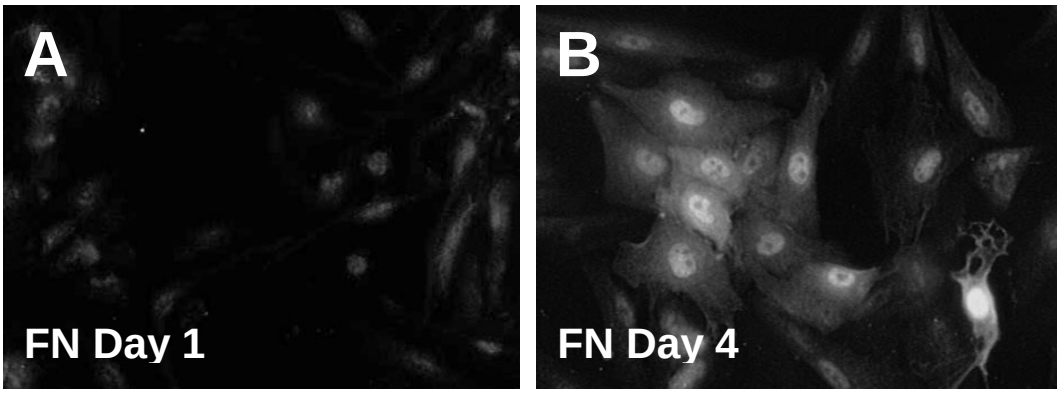

Figure 6: *Immunostaining for CRBP-1 in SMCs grown on FN in serum-free medium in primary culture. Note increased nuclear staining for CRBP-1 in almost all cells after 4 days of culture.*

Subsets of SMCs have also been described in chicken and bovine vessels [Frid et al., 1997; Topouzis and Majesky, 1996]. However, since distinct SMC phenotypes have not yet been identified in humans, the significance of these observations for the biology of human lesion development is difficult to predict. Intimal hyperplasia has also been proposed to develop from adventitial fibroblasts. Apparently, this may take place after severe vessel injury with rupture of the media and has mostly been described after oversized balloon dilatation of porcine coronary arteries [Scott et al., 1996; Shi et al., 1996]. This concept has recently been challenged since also after severe vessel injury in porcine coronary arteries, the cells of the intima express specific SMC markers, including smoothelin, which are not found in adventitial fibroblasts [Christen et al., 2001].

THE SMOOTH MUSCLE CELL

SMCs are the only cellular constituent of the arterial media. Therefore, by removing the tunica intima and tunica adventitia, one is left with an almost clean population of arterial SMCs. Utilising this principle, SMC culture became established in the early 70's [Chamley-Campbell et al., 1979] and two main techniques have been used. First, the explant method, in which the artery is freed from intima and adventitia, and cut into small pieces that are placed on a petri dish and incubated with medium supplemented with serum. After a few days, cells start to migrate out of the tissue and gradually form a confluent cell culture. This method is useful especially if tissue supply is limited. The second method (see Methods) involves enzymatic digestion of arteries. After careful removal of the intima and adventitia, the arteries are cut into small pieces and digested in an enzyme solution that breaks down the ECM so creating a suspension of free cells. The cells are washed and then cultured in serum-containing or serum-free medium. The most commonly used digestive enzyme is bacterial collagenase. If needed, the solution can be supplemented with elastase, hyaluronidase and DNase. This method yields large number of cells in a short time. Moreover, it permits studies of early changes in SMC properties, which may be relevant for the behavior of these cells in the intact vessel. In order to subculture SMCs, primary cultures are grown to confluency, detached from the surface by trypsin, and then reseeded in cell culture dishes or flasks in serum-containing medium.

During development of the arterial wall, SMCs express a range of phenotypes [Hungerford and Little, 1999]. In the adult artery, SMCs reside in a differentiated and so-called 'contractile' phenotype. Their primary function is to contract and to participate in the regulation of vessel tone [Campbell and Campbell, 1995; Gabella, 1984]. The cytoplasm in these cells is largely filled with myofilaments and organelles involved in macromolecule synthesis such as endoplasmatic reticulum (ER), Golgi apparatus and free ribosomes are small or few in number. At the other end of the spectrum are the so-called 'synthetic' cells. This phenotype is seen during

development of vessels in the embryo, in repair processes after arterial injury, and in atherogenesis [Campbell and Chamley-Campbell, 1981]. The primary function of the synthetic cells is to generate new tissue by proliferation and synthesis of ECM components. Fine structural analysis show that the cytoplasm in these cells is filled with synthetic and secretory organelles such as ER, Golgi apparatus and free ribosomes but few myofilaments [Chamley-Campbell et al., 1979]. However, also a contractile cell must have a certain level of protein synthesis, and the concept of two phenotypes is an over-simplification. More likely, a series of phenotypes exist or a gradual transformation between the two extremes takes place. When I discuss the two phenotypes (contractile and synthetic) in this thesis, I refer to cells that have predominantly characteristics of one of the two extremes. Thus, they are in most cases classified according to their ultrastructural properties and not to their function. The function of 'contractile' cells is difficult to test in a two-dimensional culture (a petri dish), although SMCs have been reported to contract in response to vasoactive agents during the first days of primary culture [Chamley et al., 1977; Mauger et al., 1975]. Instead, one could use molecular markers for either phenotype or other more specific criteria. When SMCs are established in culture, the levels of a number of cytoskeletal proteins, proposed to be biochemical markers of a more differentiated phenotype, decline [Owens, 1995; Thyberg, 1996a]. For example, SM α-actin expression decreases in cultured SMCs and instead the levels of the beta isoform increase [Gabbiani et al., 1984]. In a similar manner, decreased expression of desmin [Shanahan et al., 1993], SM myosin heavy chain [Benzonana et al., 1988], caldesmon [Birukov et al., 1993], calponin [Birukov et al., 1993; Gimona et al., 1992], SM-22 [Shanahan et al., 1993] and smoothelin [van der Loop et al., 1997; van Eys et al., 1997] have been reported. In general, detailed analyses correlating the expression of these markers to the changes in fine structure and function of SMCs in primary culture are lacking. In this thesis, I have attempted to provide information about structural components, protein expression, and signaling pathways that are associated with the transition of the cells from a contractile to a synthetic phenotype.

In terms of fine structure, SMCs which are isolated by enzymatic digestion and cultured for 1-2 days in serum-containing medium are rich in myofilaments but poor in ER, Golgi and free ribosomes, and so retain the features of contractile cells in vivo [Campbell and Campbell, 1995; Thyberg, 1996a]. In addition, the cells are quiescent and do not divide after mitogenic stimulation. However, after 2-4 days, myofilaments are gradually lost from the cytoplasm and replaced by synthetic organelles. Once the cells have modulated to a synthetic state, they start to proliferate in response to growth factor stimulation [Chamley-Campbell et al., 1981]. In order to identify factors involved in the change in phenotype under these culture conditions, Hedin and Thyberg cultured freshly isolated rat aortic SMCs under serum-free conditions on dishes precoated with different plasma protein fractions (prepared by precipitation in

cold ethanol). These experiments led to the identification of FN as a major factor in plasma responsible for the phenotypic modulation of SMCs in primary culture [Hedin and Thyberg, 1987]. The findings also introduced the ECM as an important regulator of SMC phenotype. In subsequent studies, substrates of laminin and collagen type IV were shown to retain the contractile features of the cells, thus acting opposite to FN. However, on these substrates, production of FN by the cells themselves eventually led to a change in phenotype [Hedin et al., 1988].

As earlier mentioned, a prerequisite for SMCs to respond to mitogenic stimulation is the phenotypic modulation from a contractile to a synthetic state [Chamley-Campbell et al., 1981; Thyberg et al., 1983]. Numerous mitogens have been shown to stimulate proliferation of SMCs in culture [Berk, 2001; Jackson, 1995]. These include receptor tyrosine kinase (RTK) agonists such as FGF-2 and PDGF, G-protein coupled receptor (GPCR) agonists such as thrombin and angiotensin II, and cytokines such as interleukin-1 and interleukin-6. However the role of most growth factors in SMC proliferation in vivo is difficult to predict. For example, infusion of PDGF into rats subjected to carotid injury only slightly increased SMC proliferation but greatly increased the migration of SMCs from the media to the intima during the first 7 days after injury [Jawien et al., 1992]. This indicates that although PDGF is a strong mitogen for SMCs in vitro, its major role in vivo is to promote migration rather than proliferation. The most established growth factor for SMC proliferation in vivo is FGF-2. Lindner and Reidy could inhibit DNA synthesis in the arterial media by treating the animals with a neutralizing antibody against FGF-2 [Lindner and Reidy, 1991]. The large variety of growth factors that may be involved in the proliferation of SMCs in the artery wall probably leads to a redundancy that might explain the failures of clinical trials against restenosis where individual growth factors have been targeted [Moliterno and Topol, 1998].

THE EXTRACELLULAR MATRIX

The ECM of the normal vessel wall

Basement membrane (BM - also known as basal lamina) is a specialized ECM structure that remains in close proximity to the cell it was formed by. The main constituents of the BM are laminin, collagen type IV, entactin (also called nidogen) and the heparan sulfate proteoglycan, perlecan. It is found underneath all epithelial and endothelial cells and surround muscle cells, Schwann cells and fat cells. BMs serve as a mechanical support for the cells, form a barrier between tissue compartments regulating transmigration of cells and exchange of macromolecules, and act as a substrate for cell- adhesion, spreading, and migration [Timpl and Brown, 1996; Yurchenco, 1994]. In addition, the BM is important in cell growth and differentiation.

Laminin is a 850 kD heterotrimeric molecule and the main structural component of BMs [Timpl, 1996; Timpl and Brown, 1996; Timpl et al., 1979]. The three chains α, β and γ associate to a triple helical coiled coil with a cross-like structure with three short arms and one long arm. Laminins exist as several isoforms formed by the different combinations of the α, β and γ subunits. Laminin has been shown to bind to cells via integrins α1β1, α2β1, α3β1, α6β1 and α6β4 [Ayad et al., 1994].

Entactin is a 150 kD glycoprotein [Carlin et al., 1981] with three globular domains and binding sites for collagen type IV, fibulin-1 and -2, perlecan and laminin [Timpl and Brown, 1996; Timpl et al., 1983]. It is therefore believed to be involved in linking the other constituents of the BM together.

Perlecan is the major heparan sulfate proteoglycan in the BM [Costell et al., 1999]. It consists of five domains with the heparan sulfate chains attached to domain 1 [Murdoch et al., 1992; Noonan et al., 1991]. Besides its structural function in the BM, it may also tranduce signals to the cell [Costell et al., 1999]. Similar to other heparan sulfate proteoglycans, perlecan can bind and store growth factors which may be important for growth stimulation or inhibition [Mathiak et al., 1997; Timar et al., 1992]. Perlecan also endows the membrane with an electrostatic negative charge that may partly explain the BM's filtering properties [Miettinen et al., 1986].

Type IV collagen is found exclusively in BMs where it provides structural integrity. Unlike type I and III collagen (see below) it forms a three-dimensional network, providing a scaffold for the assembly of the other BM components. The filtering properties of the BM may partly depend on the meshwork-like structure formed by type IV collagen [Ayad et al., 1994]. Small quantities of type VIII collagen has also been found in BMs [Wight, 1996].

Interstitial matrix

Fibronectin is a ~440 kD dimeric glycoprotein widely distributed in different ECMs. There are 3 types of polypeptide domains (I, II and III) in FN. Each chain consists of 12 type I domains, 2 type II, and 15-17 type III domains with the alternatively spliced segments ED-A and ED-B (ED stands for extra domain). Adhesion of cells to FN is dependent on the Arg-Gly-Asp (RGD)-containing cell-binding sequence present in domain III and can be inhibited by soluble RGD peptides [Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984]. In addition, FN has binding sites for collagen, fibrin and heparin [Magnusson and Mosher, 1998]. 'Cellular FN' (produced locally in the tissues) contains varying amounts of ED-A and ED-B while 'plasma FN' (synthesized by hepatocytes) does not contain either of these extra domains [Magnusson and Mosher, 1998]. The α5β1 integrin is the main receptor for FN in SMCs [Bottger et al., 1989; Magnusson and Mosher, 1998]. Adhesion of these cells to FN can be inhibited by soluble RGD peptides [Hedin et al., 1989]. Unlike laminin, FN

does not spontaneously polymerize in physiological fluids and FN matrix assembly is a cell-mediated event. Initially FN binds to integrins on the cell surface and the molecules are converted into fibrils at specialized sites called LAMMs (molecules of large apparent molecular mass; [Peters and Mosher, 1987]). In the normal vessel wall, there are significant amounts of FN, but it lacks the ED-A or ED-B domains [Labat-Robert et al., 1985].

Elastin is the major constituent of elastic fibers that form a network in the vascular wall. The soluble precursor of elastin, tropoelastin exists as a single polypetide chain but has several isoforms due to alternative splicing. The principle function of elastin is to provide resilience to the tissue [Vrhovski and Weiss, 1998]. Elastin levels vary in different arteries and are more prominent in larger arteries [Clark and Glagov, 1985].

Collagen is a major component of all ECMs. It consists of a triple helix of polypeptide chains (referred to as α chains) and at least 20 different α chains have been identified, forming almost 20 different types of collagen [Byers, 2000; Miller and Gay, 1987]. Collagen types I and III are the predominant collagens in the vascular interstitial matrix. In addition smaller quantities of type VI collagen exist in the normal vessel wall. Type I and III collagen are organized into fibrils while type VI collagen forms a network [Wight, 1996].

Proteoglycans are protein-polysaccharide complexes that consist of a protein core to which one or more glycosaminoglycan (GAG; chondroitin sulfate, dermatan sulfate, heparan sulfate and keratan sulfate) chains are covalently attached. Hyaluronan, another GAG, exists as a free molecule in the ECM [Wight, 1996]. The major proteoglycans in the vascular interstitial matrix are versican (~1000 kD), decorin (90-140 kD) and biglycan (~150-240 kD). Decorin associates with collagen fibrils and regulate their diameter, while versican interacts with hyaluronan to form large aggregates that bind water and occupy the interstitial space [Wight, 1996].

The ECM of the diseased intima

The BM surrounding SMCs has been proposed to be a negative regulator of SMC growth [Hedin et al., 1999; Newby and Zaltsman, 1999]. This theory is supported by observations in chicken embryos. Early during during vessel formation, SMCs proliferate in a matrix devoid of laminin but rich in FN. On the other hand, laminin is expressed on embryonic day 8 as SMCs stop proliferating and the vessels mature [Hungerford and Little, 1999; Risau and Lemmon, 1988]. We have studied the distribution of laminin and FN around SMCs after balloon injury of the rat carotid artery. Uninjured arteries contained laminin around SMCs in the media. After injury, laminin staining was replaced by staining for FN. The presence of FN around the cells correlated temporally and spatially to SMC activation, migration and proliferation. Five weeks after injury, laminin staining reappeared around SMCs in the intima at the

same time as the fine structure of these cells indicated that they had reverted to a quiescent and contractile phenotype [Thyberg, 1998a; Thyberg et al., 1997]. High levels of FN are found in intimal thickenings in atherosclerosis, transplant arteriosclerosis and postangioplasty restenosis [Clausell and Rabinovitch, 1993; Molossi et al., 1993]. Moreover, not only 'plasma FN' but also locally produced FN containing the ED-A and ED-B domains are expressed in thickened intimas and atherosclerotic lesions but not in the media [Glukhova et al., 1989]. Similarly, these FN isoforms have been observed to be induced after arterial injury in animal models [Bauters et al., 1995; Dubin et al., 1995]. Versican and hyaluronan, which promote SMC migration and proliferation in vitro [Evanko et al., 1999], also accumulate in the intima [Wight, 1996]. Other potential ECM components that may be involved in the pathogenesis of atherosclerosis and intimal thickening are tenascin [Hedin et al., 1991; Wallner et al., 1999] and osteopontin [Giachelli et al., 1995], both of which have been shown to be upregulated in human atherosclerotic lesions, and after angioplasty in humans and rats.

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc dependent enzymes that degrade ECM components under physiological and pathological conditions. Almost 30 members of this family have been identified. They are divided into five different groups based on their structure and substrate specificities; collagenases (MMP-1, 8, and 13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, -11), membrane-type MMPs (MT-MMPs; MMP-14-17) and others [Yong et al., 2001]. MMP activity is regulated by the presence of activating enzymes such as urokinase-type plasminogen activator (uPA) that cleaves the proenzyme to its active form, and the presence of TIMPs [Woessner and Nagase, 2000]. In addition, reactive oxygen species and MT-MMPs have been shown to regulate MMP activity [Rajagopalan et al., 1996; Sato et al., 1994]. MMP-2 and MMP-9, synthesized by SMCs and macrophages, have been shown to be expressed in response to vascular injury [Zempo et al., 1994]. In atherosclerotic plaques, MMP-1, MMP-3, MMP-9, and MMP-12 have been identified and proposed to be involved in plaque rupture [Newby and Zaltsman, 1999]. TIMPs have also been detected in atherosclerotic tissue and often in higher levels than in non-atherosclerotic tissues suggesting, that they may serve as a protective mechanism for the tissue [Fabunmi et al., 1998].

CELL-MATRIX INTERACTIONS

After this description of SMCs and the ECM, I would now like to shift the focus to interactions between cells and the ECM. I will start by describing the structural entities that mediate attachment of cells to the ECM and then continue with a few of the intracellular signaling pathways that are believed to be activated by these

interactions. In the last part of this section, I will describe how cell-matrix interactions regulate cell cycle progression and cell shape.

Integrins

Integrins are a family of cell surface receptors that mediate binding of cells to the ECM. They are transmembrane heterodimeric glycoproteins consisting of α and β subunits. Each subunit has a large extracellular domain, a transmembrane domain and a short cytoplasmic domain (except for the β4 subunit) [Aplin et al., 1998; Burridge and Chrzanowska-Wodnicka, 1996]. At least 16 different α subunits and 8 different β subunits have been identified, forming more than 20 integrins [Hynes, 1992; Rosales et al., 1995]. The extracellular domain binds ECM proteins with a specificity that is determined by the pairing of the different α and β subunits. Some integrins, such as the classic FN receptor $\alpha_5\beta_1$, binds only to one ECM component while many others bind multiple proteins for example $\alpha_v\beta_3$ which interacts with thrombospondin, laminin and fibrinogen [Plow et al., 2000]. The cytoplasmic tails of the integrin subunits have binding sites for cytoskeletal proteins, signaling molecules and adaptor proteins, which link the ECM to the actin cytoskeleton and to intracellular signaling cascades. Mutational analysis has shown that the β subunit is critical for integrin function, and deletion of the β subunit prevents the localization of the integrin to focal adhesions (FAs). Since deletion of the α subunit promotes integrin recruitment to FAs, it has been proposed that the α subunit is inhibitory on the β subunit, and this inhibition is removed upon appropriate ligand binding [Aplin et al., 1998].

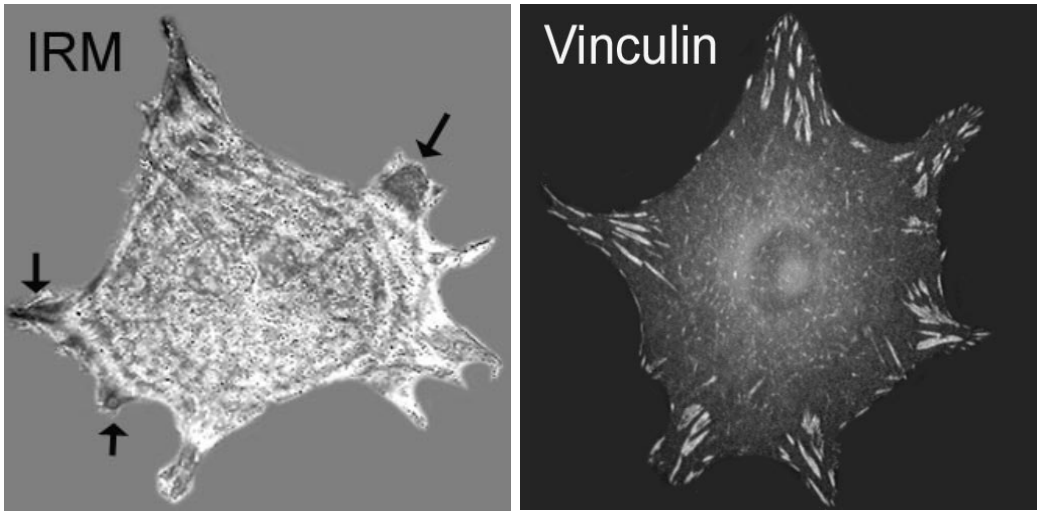

Figure 7: *Demonstration of two different methods to visualize FAs. Left panel shows a cell studied by IRM where the dark areas are in close contact with the surface the cells are grown on. FAs are indicated by arrows. In the right panel, immunostaining for vinculin, an intracellular FA protein, demonstrate the distribution of this protein in FAs.*

Focal adhesions

FAs are specialized attachment sites where the cell interacts with the ECM. FAs consist of integrin clusters that bind to ECM components outside the cell and protein complexes that interact with cytoplasmic domains of integrins in the interior [Burridge and Chrzanowska-Wodnicka, 1996]. FAs are easily detected in adherent cells in culture but have rarely been observed in vivo. The analogy to FAs observed in tissues are the dense plaques on the intracellular side of the plasma membrane in SMCs. These structures are also believed to mediate cell anchorage, cytoskeletal organization, and to be involved in intracellular signaling [Hai, 2000]. FAs are seen as dark patches in the cell membrane on the bottom of cells using interference reflection microscopy (IRM; [Alberts et al., 1994]). Other methods include staining for vinculin (a component of FAs), which characteristically shows a punctate staining at the cell membrane (Fig. 7; [Burridge and Chrzanowska-Wodnicka, 1996]). The main cytoplasmic proteins of FAs that bind integrins to the actin cytoskeleton are talin, α-actinin, vinculin, paxillin, and tensin (Fig. 8; [Aplin et al., 1998]). It is now appreciated that FAs are not static structures but rather assemble and disassemble during the lifetime of a cell. For example, assembly of FAs takes place during cell adhesion and spreading, when new FAs form at the periphery of the cell. Disassembly, on the other hand, occurs in serum-starved cells and at the rear of migrating cells where they need to detach from the ECM as they move [Sastry and Burridge, 2000]. Besides serving as an anchorage structure for the cell, FAs are centers for signaling initiated by the ECM. Signaling molecules such as focal adhesion kinase (FAK; discussed below) and integrin-linked kinase that are directly or indirectly associated to integrins can be activated upon ligand binding to the integrin [Aplin et al., 1998; Burridge and Chrzanowska-Wodnicka, 1996; Sastry and Burridge, 2000]

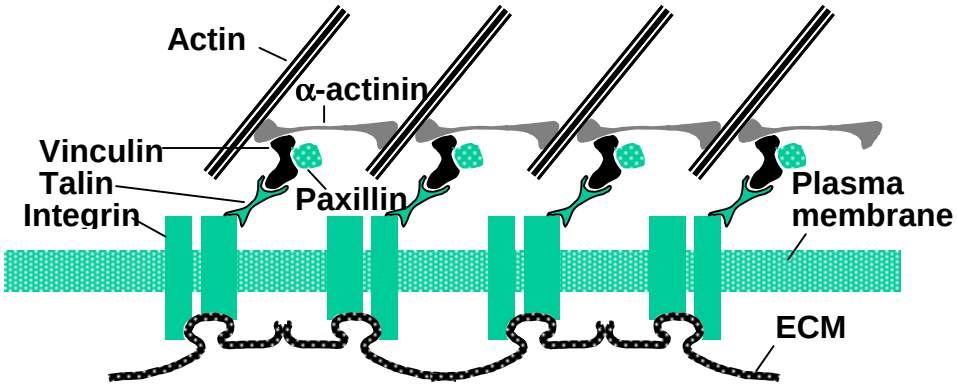

Figure 8: *Schematic illustration of FAs. Integrins bind to specific cell-binding domains in ECM proteins and cluster at the plasma membrane. Binding of specific FA proteins to the cytoplasmic tail of these receptors then facilitate actin organization.*

INTEGRIN-MEDIATED SIGNALING

Focal adhesion kinase

Focal adhesion kinase (FAK) is a 125 kD non-membrane bound protein tyrosine kinase that is tyrosine phosphorylated upon activation of most integrins. However, FAK can also be activated by non-integrin stimuli such as growth factors [Rodriguez-Fernandez, 1999; Schlaepfer et al., 1999]. The mechanism by which integrins activate FAK is not known in detail but the process requires an intact actin cytoskeleton since cytochalasin D treatment prevents FAK activation [Shattil et al., 1994]. FAK has a central kinase domain, a C-terminal domain that contains a 'focal adhesion targeting' (FAT) sequence and a N-terminal domain that may associate with the β subunit of integrins. The FAT sequence contains binding-sites for paxillin and talin, which in turn can directly bind to the β subunit of integrins and thereby mediate recruitment of FAK to FAs. FAK has six or more sites (including Tyr397, Tyr407, Tyr576, Tyr577, Tyr861, and Tyr925), which are phosphorylated upon integrin activation. Phosphorylation at Tyr576 and Tyr577 is required for maximum kinase activity. Integrin activation and autophosphorylation at Tyr397 permits binding of the signaling molecules, Src or Fyn. Src can then phosphorylate other FA proteins including $p130^{CAS}$, paxillin, tensin and FAK itself at Tyr407, Tyr 576, Tyr577, Tyr861 and Tyr925. This 'second wave' of phosphorylation creates a binding site at Tyr925 for the adaptor protein growth factor receptor-binding protein 2 (Grb2), which is involved in the activation of Ras in the Ras-Raf-Mek-ERK pathway (described below). Other downstream targets of FAK include the PI-3 kinase pathway, $p130^{CAS}$ and paxillin [Aplin et al., 1998; Schlaepfer et al., 1999].

Studies using FAK-deficient cells isolated from FAK-/- mice have demonstrated that FAK is not critical for FA formation. Instead, these cells displayed decreased motility on FN, indicating that FAK is necessary for cell migration [Ilic et al., 1995]. An alternatively spliced isoform of FAK, FRNK, has a natural inhibitory activity on FAK [Richardson and Parsons, 1996; Schaller et al., 1993; Schlaepfer et al., 1999]. Overexpression of FRNK was recently shown to decrease intimal hyperplasia after balloon injury in rat carotid arteries suggesting a role for FAK in SMC activation in vivo [Taylor et al., 2001].

The Ras-Raf-MEK-ERK pathway

The extracellular signal regulated kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase family (MAPK), which have been shown to be involved in diverse processes such as cell growth, migration and differentiation. At least four different MAPK pathways exist, including the stress-activated protein kinase (SAPK) pathway, the p38 MAPK pathway, the ERK5 pathway, and the ERK1/2 pathway. Upon activation, ERKs are translocated into the nucleus where they

activate a series of transcription factors and thereby provide a link between the exterior of the cell and the nucleus [Lenormand et al., 1993; Treisman, 1996]. ERK1/2 are phosphorylated and activated by specific MAPK kinases (also known as MEKs), and MEK1/2 are in turn activated by MAPK kinase kinases such as Raf. Most RTKs activate the GTP-binding protein Ras which activates and recruits Raf to the plasma membrane (Fig. 9). The link between integrins and Ras/Raf is not well established but may take place via activation of Shc or FAK which can activate Grb2. Grb2 then recruits the guanine nucleotide exchange factor (GEF), SOS (Son of sevenless). These interactions bring SOS into proximity of Ras and stimulates GTP/GDP exchange. The signal is thereafter propagated downstream through the subsequent activation of Raf, MEK, and finally ERK1/2. In SMCs, ERK activation has been shown to be involved in phenotypic modulation ([Hayashi et al., 1999], paper III), migration [Bornfeldt et al., 1995; Bornfeldt et al., 1994], proliferation [Bornfeldt et al., 1995] and apoptosis [Miyamoto and Fox, 2000]. Furthermore, balloon injury of the rat carotid artery was followed by an increase in ERK phosphorylation within 1 hour, which returned to baseline after 12-24 hours [Koyama et al., 1998; Lille et al., 1997]. Administration of the MEK1 inhibitor, PD98059, immediately after injury also inhibited intimal hyperplasia indicating that ERK1/2 activity is involved in the development of the neointima [Koyama et al., 1998]

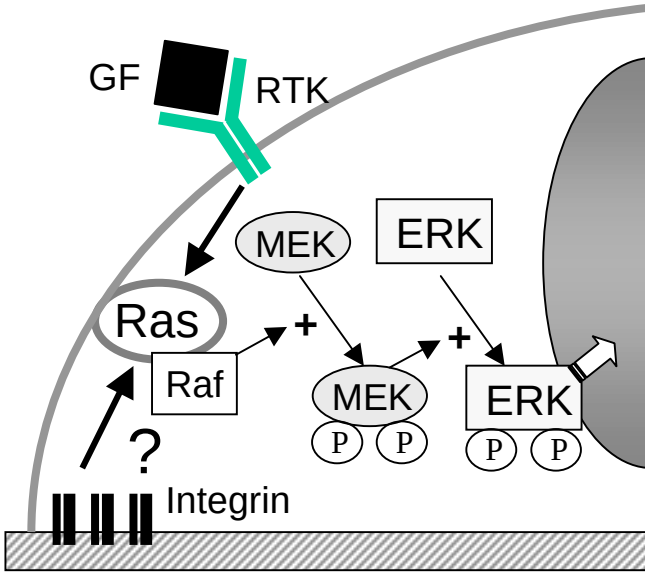

Figure 9: *Schematic illustration of the Ras-Raf-MEK-ERK pathway. Binding of growth factor (GF) to a receptor (RTK) or integrin ligation facilitates activation of Ras, which bind and activate Raf at the plasma membrane. Raf can thereafter phosphorylate and activate MEK, which in turn activates ERK. Phosphorylated ERK is then translocated into the nucleus where transcription factors are targeted.*

Rho family proteins

The Rho family proteins constitute a subgroup of the Ras superfamily of GTPases, which also includes the Ras, Rap and Rab proteins. The most well characterized members of the Rho family are RhoA, Rac1 and Cdc42. These proteins have specific actions on the cytoskeleton: RhoA is involved in stress fiber formation and FA assembly, Rac1 in membrane ruffling and lamellopodia formation, and Cdc42 in

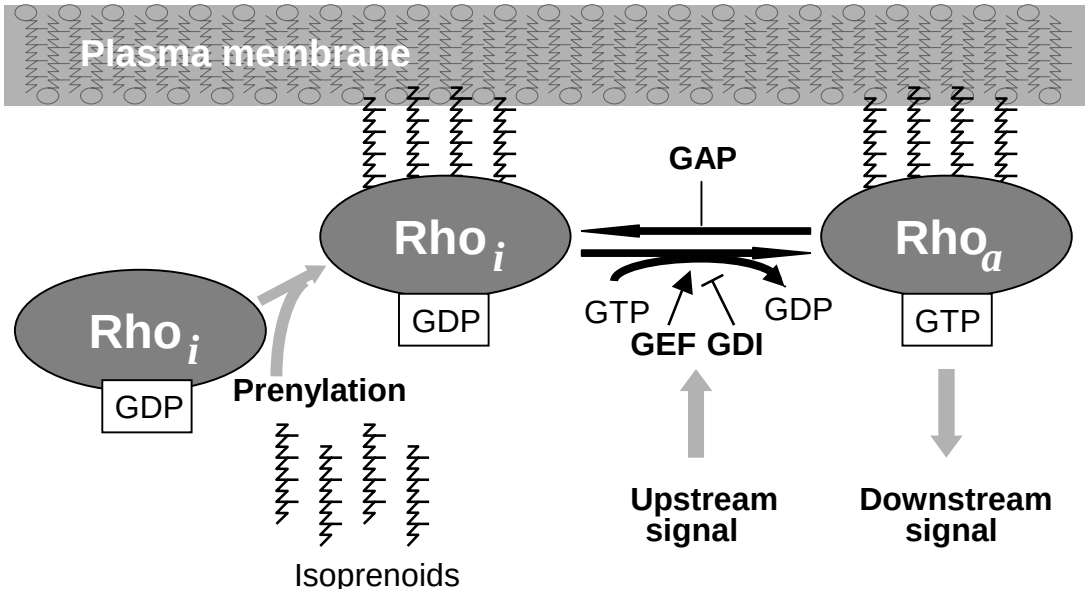

Figure 10: *Schematic illustration of Rho activation. Inactive Rho (Rho_i) in the cytosol is prenylated through the attachment of isoprenoid groups such as geranylgeranyl, and associates with the plasma membrane. In this form, Rho is the target for GEFs, activated by various stimuli (upstream signal), which facilitate the exchange of GDP to GTP. This process activates Rho (Rho_a), which thereafter can activate downstream effectors. GDP-GTP exchange is inhibited by GDIs, which prevent the dissociation of GDP from Rho, and GAPs deactivate Rho.*

filopodia formation [Bishop and Hall, 2000]. Each of these proteins act as a molecular switch, cycling between an active GTP-bound and an inactive GDP-bound state (Fig. 10). In the active state, they are able to bind and activate their effector proteins, initiating a downstream response. Cycling between the active and inactive states is regulated by numerous proteins. GEFs facilitate the exchange of GDP for GTP while the GTP-ase activating proteins (GAPs) stimulate hydrolysis of GTP to GDP. In the inactivated state, the proteins are in the cytoplasm bound to guanine nucleotide dissociation inhibitors (GDI) that prevent the spontaneous conversion of GDP to GTP. However, the precise significance of GDIs is not understood [Bishop and Hall, 2000]. Like other members of the Ras superfamily, the Rho proteins interact with the cytoplasmic side of cellular membranes where they perform their role as regulatory proteins. Membrane association requires prenylation, which is a modification of the carboxy terminal end of the protein with an isoprenoid, in the case of the Rho proteins geranylgeranyl. Ras proteins, on the other hand, are modified with farnesyl [Seabra, 1998]. Therefore, the availability of the isoprenoids, which are metabolites of mevalonate, an intermediary in cholesterol metabolism (see Results and Discussion, Fig. 15), can affect the activity of the small GTP-binding proteins [Aktories, 1997; Laufs and Liao, 2000]. The mechanism by which integrins activate Rho proteins has not been elucidated but it may involve the activation of GEFs [Sastry and Burridge, 2000]. One known example is the RhoGEF, Vav1, in hematopoetic cells, which is

tyrosine phosphorylated upon integrin clustering [Miranti et al., 1998; Zheng et al., 1996]. The downstream targets of the Rho family proteins are numerous, indicating a central role in different cellular processes like cell adhesion, spreading and gene transcription [Bishop and Hall, 2000]. In addition to its role in cytoskeletal organization, recent work has shown that Rho and Rac are also involved in SMC proliferation [Laufs et al., 1999; Negre-Aminou et al., 2001]. Furthermore, an inhibitor (Y-27632) of the ROCK family of kinases [Uehata et al., 1997], which include a number of downstream targets of Rho, has been shown to prevent the development of neointima in the rat balloon carotid injury model [Sawada et al., 2000].

Caveolae and caveolin

Caveolae are small invaginations in the plasma membrane that have been suggested to be involved in signaling and cholesterol trafficking [Schlegel and Lisanti, 2001]. The major coat protein on the cytoplasmic surface is caveolin. Three caveolin isoforms have been cloned [Schlegel and Lisanti, 2001]. Most of our knowledge about signaling is confined to studies with caveolin-1. Caveolin-2 interacts with caveolin-1 and caveolin-3 has been detected in muscle tissues and astrocytes only. Caveolin-1 is a 22 kD protein with a central hydrophobic domain which anchors the protein to the cell membrane and two large cytoplasmic domains [Rothberg et al., 1992; Schlegel and Lisanti, 2001]. Caveolin-1 is believed to take part in the transport of cholesterol from the ER to the caveolae membrane and the efflux of excess free cholesterol from the cells [Fielding and Fielding, 2000; Fielding and Fielding, 1995]. In the early 90's Lisanti and coworkers discovered that many of the proteins found in caveolae were signaling molecules and proposed the 'Caveolae Signaling Hypothesis' [Lisanti et al., 1994]. According to this idea, caveolae are a sub-compartment of the plasma-membrane where signal transduction events occur and the concentration of signaling proteins may provide an environment for rapid cross-talk between different signaling modules and more efficient signal transmission. Furthermore caveolin-1 was found to bind to various signaling molecules via a 20 amino-acyl residue domain, termed the caveolin scaffolding domain. This domain had been shown to recognize motifs on diverse signaling molecules such as RTKs, MEK, ERK, Src, Fyn and eNOS [Couet et al., 1997]. Generally, caveolin-1 inhibits the activity of the signaling molecules it binds. Interestingly, several integrins have been found to bind caveolin-1, which may be necessary for integrin clustering. In support of this notion, inhibition of caveolin expression has been shown to prevent FA assembly [Wary et al., 1998; Wei et al., 1999].

Cell-matrix interactions in the regulation of cell-cycle progression

The eukaryotic cell cycle is controlled by periodic changes in the expression and activity of cyclins and cyclin-dependent kinases (cdks). Initially, proliferation stimuli lead to the induction of cyclins D, E, and A which bind to the appropriate cdks and regulate progression from G1 to S. Later, cyclin B associates with cdk1 and regulates the G2-M traverse. Cell cycle progression is also regulated by the levels of cyclin-dependent kinase inhibitors (CKIs) such as p15 ^{INK4B}, p16^{INK4A}, p21^{CIP1} and p27^{KIP1} [Sherr, 1993; Sherr and Roberts, 1999]. It has long been known that non-neoplastic cells are anchorage-dependent i.e. require adhesion to a solid ECM substrate for cell proliferation (Fig. 11). Recent studies have provided some insight into the molecular mechanisms involved [Assoian and Schwartz, 2001; Roovers et al., 1999; Weber et al., 1997]. ERK1/2 activation is probably the most well studied signaling pathway involved in cell proliferation. The duration rather than the magnitude of ERK1/2 activation seems to be important for cyclin D1 induction and subsequent entry into the S-phase. Growth factor activation of cells in suspension or adhesion of cells to a matrix in the absence of growth factors could both activate ERK1/2 but only for a short duration, which was not sufficient for cyclin D1 induction. However, the combination of growth factor stimulation of cells on a matrix (i.e. adherent cells) led to sustained ERK activity, cyclin D1 induction, and progression into the S-phase [Assoian and Schwartz, 2001; Roovers and Assoian, 2000]. Simply activating ERK in suspended cells does not always overcome the need for integrin activation,

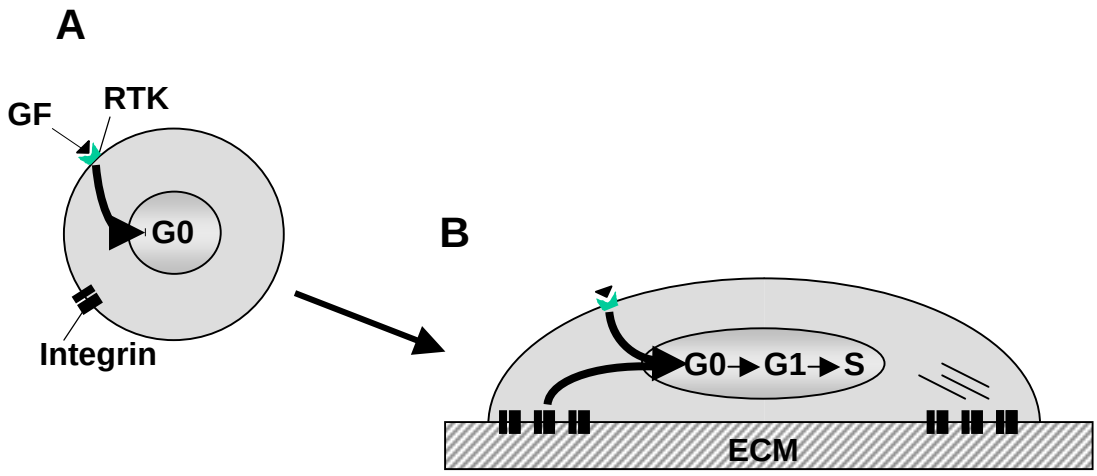

Figure 11: *Schematic illustration of the cooperative effect of the ECM and growth factors (GF) in cell cycle entry and progression in non-transformed cells. (A) In cells in suspension, without anchorage to the ECM, binding of GF to receptors (receptor tyrorosine kinases; RTK), elicit intracellular signals to the nucleus but in the absence of integrin activation by the ECM, the cell does not enter the cell cycle and remain in G0. (B) After adhesion to the ECM, integrin clustering, and cytoskeletal organization, integrin-dependent signaling pathways cooperate with GF-mediated signaling and together these pathways facilitate G0 exit and further cell cycle progression into S-phase.*

suggesting that other mechanisms are involved [Le Gall et al., 1998]. Other probable signaling molecules involved are the CKIs p27 and p21 and the Rho proteins. Some groups have shown that cyclin D1 induction and S-phase entry are dependent on the activation of Rho family proteins independent of the level and duration of ERK activation [Danen et al., 2000; del Pozo et al., 2000]. Cell adhesion to the ECM has also been shown to promote cell cycle progression by decreasing levels of or redistributing p27 and p21 and hence increasing cdk2 activity [Fang et al., 1996; Koyama et al., 1996; Kuzumaki and Ishikawa, 1997; Radeva et al., 1997]. In addition to adhesion per se, the choice of matrix also affects cell cycle progression [Huang et al., 1998; Huang and Ingber, 1999; Junker and Heine, 1987]. The effect of different matrices on cell cycle progression may depend on their ability to affect cell shape (refers to cell spreading in this context). To support this idea, Huang and coworkers have shown that by physically limiting cell spreading on a matrix of FN an increase in p27 levels and a decrease in cyclin D1 levels was observed, arresting the cells in G0-G1. Hence, various mechanisms exist by which the ECM can affect cell-cycle progression and the exact mechanisms in different cell types and tissues may vary.

AIMS

A critical and fundamental step in the development of intimal hyperplasia is the activation of SMCs. Previously, it was shown that when SMCs are cultured on FN, their fine structure resembles that seen in synthetic cells in the vascular wall after vascular injury or during atherosclerosis. This suggested that changes in the ECM could be involved in SMC activation. In support of this notion, FN has been shown to be upregulated after vascular injury in animal models [Pickering et al., 2000; Thyberg et al., 1997]. However, our initial strategy to inhibit the interaction of SMCs with FN did not succeed due to the lack of specific functional antibodies against FN. The use of blocking peptides was possible but affected adhesion efficiency to such a degree that work using primary cultures of SMCs was not possible. We therefore turned our attention to the downstream events of integrin activation.

The principal objective of this thesis was to understand the signaling mechanisms that regulate the activation of SMCs by the ECM with specific focus on FN. We studied the main pathways shown to be activated by integrins in other cell types to see their role in SMC activation. In addition, we wanted to test the feasibility of targeting these signaling pathways using a suitable drug.

MATERIALS AND METHODS

PRIMARY CULTURE OF RAT AORTIC SMOOTH MUSCLE CELLS

Although in vitro culture of SMCs was described as early as in 1913/1914 [Champy, 1913/1914], primary culture of SMCs was first well characterized in the 1970's [Chamley-Campbell et al., 1979]. Campbell and coworkers described the change in structure and function of arterial SMCs isolated by enzymatic digestion when cultured in the presence of serum. In order to study the effects of different matrices on SMCs, our laboratory has developed a model where SMCs are isolated from rat aortas by enzymatic digestion and cultured on different matrix substrates under serum-free conditions [Thyberg, 1996b; Thyberg et al., 1990]. We have previously shown that primary culture of SMCs on FN has similar effects on SMC morphology as culture in serum [Hedin et al., 1988; Hedin and Thyberg, 1987]. Aortas from 300-400g male Sprague Dawley rats are excised and the adventitia carefully removed. The arteries are cut open longitudinally, endothelial cells gently scraped off the luminal surface of the vessel, which is then cut into small pieces and digested with bacterial collagenase at 20°C. After 8-12 hours, the cell suspension is filtered through a nylon filter and the cells counted using an electronic cell counter. About 2- 4 million cells are isolated from a rat aorta. The cells are suspended in Ham's F-12 medium containing 0.1% bovine serum albumin (BSA) and seeded in plastic dishes (~50,000 cells/cm^2) precoated with matrix substrates. An adhesion efficiency of about 75% with FN and 30% with laminin was achieved [Hedin et al., 1988]. The primary cultures are thereafter kept in F-12/0.1% BSA supplemented with 10 mM of HEPES and TES, pH 7.3, 50 µg/ml of L-ascorbic acid and 50 µg/ml of gentamycin sulfate [Thyberg et al., 1990]. Freshly isolated SMCs cultured on FN spread rapidly, whereas cells on laminin remained spindle-shaped during the first 4 days of culture. Thereafter, cells on laminin spread and resembled cells grown on FN as earlier described [Hedin et al., 1988]. The medium is changed daily and SMCs cultured on FN remain viable for more than a week with a detecable apoptotic index of less than 10% (Paper V)**.** In order not to confuse the reader, I would like to point out that primary culture in this thesis refers to freshly isolated SMCs grown in vitro. After the first trypsinisation and reseeding of the cells, they are referred to as passaged cells. This is in contrast to many authors who refer to all mortal cell cultures as primary cultures. Passaged cells were set up by growing primary cultures to confluency in 10% fetal calf serum (FCS) in F-12, followed by treatment with 0.1% trypsin in magnesium- and calcium-free phosphate-buffered saline (PBS) containing EDTA. The cells were then rinsed and set up in new dishes.

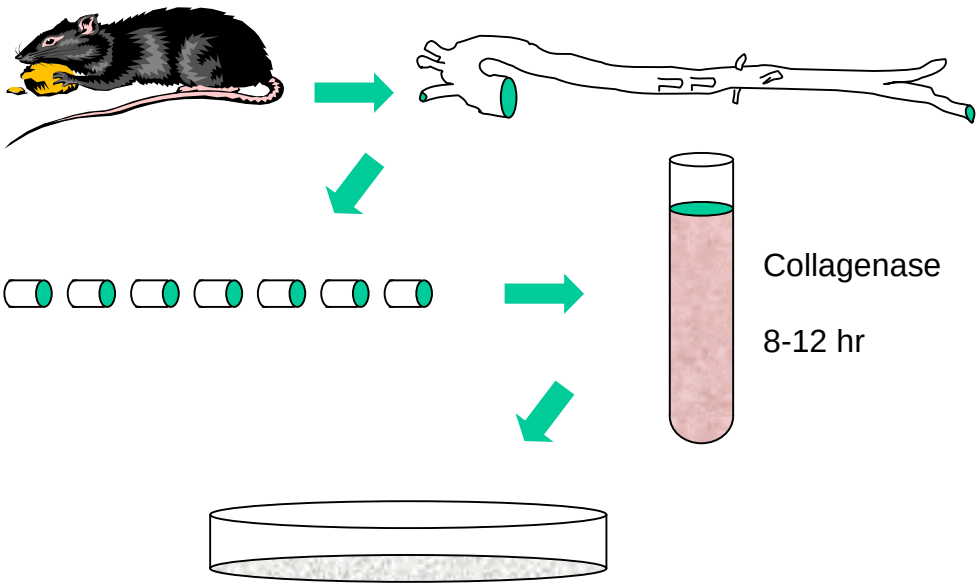

Figure 12: *Primary culture of rat SMCs. The aorta is dissected from male Sprague-Dawley rats, cut in pieces and digested overnight in collagenase. The cell suspension is thereafter filtered, rinsed and the cells seeded in petri dishes.*

PREPARATION OF CELL CULTURE SUBSTRATES

Interactions between freshly isolated SMCs and ECM proteins were studied by allowing the cells to adhere to matrix-coated cell culture dishes. Human plasma FN [Engvall and Ruoslahti, 1977] and mouse laminin derived from Engelbreth-Holm-Swarm sarcoma (laminin-1**;** [Timpl et al., 1979]) were purchased from Gibco. Poly-D-lysine was obtained from Sigma. The optimal concentrations for adhesion of SMCs in primary culture to FN and laminin have been previously determined [Hedin et al., 1988]. Culture substrates were prepared by dissolving FN (10-20 µg/mL), poly-D-lysine (50 µg/ml) or laminin (20 µg/mL) in PBS and allowing the proteins to adsorb to the bottom of plastic dishes or to glass coverslips for 16 hours at 20°C. Non-specific binding sites were blocked with BSA.

WESTERN BLOTTING

Immunoblotting (also referred to as Western blotting) was used to study changes in expression of proteins involved in SMC activation. SMCs cultured in petri dishes were lysed and protein concentrations determined using either the Bio-Rad protein assay (Bio-Rad Laboratories) or the Pierce BCA protein assay (Pierce, Rockford, IL, USA). Equal amount of proteins were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5–15%). The proteins were then electrophoretically transferred to nitrocellulose membranes, in a wet transfer system, and non-specific binding blocked by incubating with dry-milk or BSA-containing buffers. The membranes were incubated overnight with primary antibodies (see Table 1). After washing, the membranes were incubated with the appropriate

secondary antibody conjugated with horseradish peroxidase or alkaline phosphatase. Chemilumiscent or chromogenic substrates were used to visualize reactive bands.

IMMUNOPRECIPITATION

In order to analyze the phosphorylated state of FAK during SMC activation, we immunoprecipitated FAK using a mouse monoclonal antibody. In paper I, protein A-sepharose was used to pull down the protein. Since protein A binds only weakly to mouse IgG_1 antibodies, incubation with a secondary rabbit antibody that bound to the primary mouse antibody was necessary. In paper V, this step could be excluded by using protein G-sepharose that strongly binds to mouse IgG_1 antibodies. The immunoprecipitates were solubilized in SDS sample buffer and run on 7-7.5% SDS-PAGE and immunoblotting performed using antibodies against phosphotyrosine (4G10, PY20).

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The expression and subcellular localization of proteins involved in SMC activation was studied by immunofluorescence microscopy. Cells were cultured on glass coverslips and fixed at different time points in 2-4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and aldehyde groups reduced by treatment with 0.15 M glycine. Non-specific binding was blocked by incubating the cells with BSA- or serum-containing PBS. After incubation with a primary antibody overnight at 4°C, the coverslips were washed and incubated with secondary antibodies conjugated with either fluorescein isothiocyanate (FITC) or rhodamine for 1 hr at 20°C. The specimens were rinsed with PBS, mounted in Vectashield, an aqueous medium with anti-fading properties (Vector, Burlingame, CA), and studied using a microscope with epifluorescence optics.

In paper III, immunofluorescence microscopy was also performed on freshly isolated rat aortic SMCs prior to seeding on matrix substrates. These cells were suspended in 0.1% BSA in medium F-12 after collagenase digestion, cytospinned to glass slides, and stored at -70°C. Immunostaining with the phosphospecific antibody against active ERK1/2 required the use of Tris-buffered saline (TBS) as a buffer instead of PBS. In paper IV, we used confocal laser scanning microscopy (CLSM) to be able to better distinguish between cyclin D1 positive and negative cells. CLSM provides better optical resolution and shorter exposure times and decreased bleaching. The fluorochrome used was Cy-2 and nuclei were counterstained with propidium iodide.

IN SITU TUNEL ANALYSIS

In order to study cell death by apoptosis, we used an in situ TdT-mediated dUTP Nick-End labeling (TUNEL; Boehringer Mannheim) assay combined with electron microscopic analysis (described below). The TUNEL assay is based on the fact that

during apoptosis, prior to DNA cleavage, endogenous nucleases create a number of DNA strand breaks. To detect these breaks, SMCs fixed in 4% formaldehyde were digested with proteinase K and incubated with biotinylated dUTP and terminal deoxyribonucleotide transferase (TdT). The TdT enzyme catalyzes the binding of dUTP to the strand breaks. A streptavidin-Cy2 complex was used to detect the presence of apoptotic cells and the slides examined in an immunofluorescence microscope [Zhivotovsky et al., 1999]

DETERMINATION OF S-PHASE ENTRY

Phenotypic modulation of SMCs in primary culture is associated with a successive increase in competency to respond to growth factor stimulation [Chamley-Campbell et al., 1979; Hedin et al., 1988; Hedin and Thyberg, 1987]. In order to analyze the cell cycle phase of SMCs and quantify the cells that entered S-phase, the following methods were used:

^{3}H-thymidine labeling and detection by autoradiography

Freshly isolated SMCs were cultured on matrix substrates on glass coverslips under different conditions. After regular time intervals the cells were exposed to 1-2 μCi/ml ^{3}H-thymidine (Amersham Pharmacia Biotech) for 24 hours and some of the cultures were stimulated with growth factors during the same time period. After fixation in 3% cacodylate-buffered glutaraldehyde, rinsing in buffer, dehydration in ethanol and air-drying, the coverslips were mounted on slides and dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY). After exposure at 4°C for 2 days, the specimens were developed in Kodak D-19, fixed, and stained with 1% methylene blue. The labeling index was determined by counting at least 300 cells in each specimen. This method is highly reliable but time-consuming due to the manual microscopic counting and the long exposure times involved.

^{3}H-thymidine labeling and detection by scintillation counting

SMCs were cultured on 24-well plates precoated with FN and incorporation of ^{3}H-thymidine was performed as described above. The cells were rinsed with cold PBS and macromolecular matrial precipitated by incubation with ice-cold 5% trichloroacetic acid (TCA). The plates were then washed with ice-cold 5% TCA and the samples solubilized in 0.1 M NaOH. The plates were shaken vigorously for 30 minutes at room temperature and aliquots were mixed with 4 ml of scintillation fluid and counted in a scintillation counter. Estimation of DNA synthesis by scintillation counting is faster than by autoradiography, however, cytoplasmic pools of thymidine present as an extra source of error.

Estimation of DNA content using flow cytometry

Freshly isolated SMCs were seeded in matrix-coated petri dishes and incubated for various intervals. The cells ($0.5 – 1x10^{6}$) were then trypsinized, washed and

resuspended in 4% formalin in PBS overnight at 4°C. After centrifugation, the resulting pellet was fixed with 95% ethanol and rehydrated in water. The cells were stained with DAPI and analyzed in a flow cytometer with an ultraviolet source [Castro et al., 1993]. Flow cytometry enables a rapid analysis of the DNA content in individual cells.

ELECTRON MICROSCOPY

Phenotypic modulation of SMCs is characterized by changes in cellular ultrastructure. Typical characteristics of synthetic cells include a well developed ER and Golgi apparatus whereas in contractile cells, the cytoplasm is mainly filled with myofilaments [Chamley-Campbell et al., 1979; Thyberg, 1996b; Thyberg et al., 1983]. Electron microscopy was used to determine the phenotypic state, apoptosis and number of caveolae in contractile and synthetic cells. SMCs were cultured in petri dishes under different conditions, fixed, dehydrated and embedded in low-viscosity epoxy resin. Thin sections were cut on an ultramicrotome and examined in a transmission electron microscope. To follow the structural transformation of the SMCs, one or two non-overlapping sections from each culture was scanned and midsagittal sections through the central parts of the cells (extending from the nucleus toward the periphery) were photographed at a final magnification of 15,000 – 25,000x [Thyberg et al., 1990]. Cells were characterized as either contractile or synthetic according to the criteria stated above and described in earlier quantitative studies in our laboratory [Thyberg et al., 1983]. Alternatively, the volume density of different organelles were determined by morphometric analysis [Weibel, 1969]. Apoptosis is an orderly process whereby cells shrink in size to almost half their volume, condense their nuclear chromatin and fragment into small membrane-bound structures called apoptotic bodies [Kerr et al., 1995; Saraste, 1999]. On the basis of these criteria, cells analyzed by electron microscopy and found to be markedly shrunken with condensed nuclei, destroyed organelles in the cytoplasm but enclosed in intact cell membranes were classified as apoptotic.

Table 1: *Primary antibodies used for immunoblotting (IB), immunofluorescence (IF) and immunohistochemistry (IHC).*

Antigen	Clone	Host	Dilution	Source
Caveolin	C060	Mouse (M)	1:500 (IB)	Transduction Laboratories, Kensington, KY, USA
Cyclin A	H-432	Rabbit (P)	1:100 (IF) 1:1000 (IB)	Santa Cruz Biotechnologies, Santa Cruz, CA, USA
Cyclin D1	72-13G	Mouse (M)	1:100 (IF, IHC) 1:1000 (IB)	Santa Cruz Biotechnologies
Cyclin D3	D-7	Mouse (M)	1:1000 (IB)	Santa Cruz Biotechnologies
ERK (phosphorylated)	-	Rabbit (P)	1:100 (IF) 1:1000 (IB)	New England Biolabs, Beverly, MA, USA
ERK	- -	Rabbit (P) Rabbit (P)	1:1000 (IB) 1:1000 (IB)	New England Biolabs R. Seger, Seattle, WA, USA
FAK	Clone 77	Mouse (M)	1:1000 (IB)	Transduction Laboratories
Golgi α-mannosidase II	-	Rabbit (Antisera)	1:300 (IF)	K.W. Moremen, Athens, GA and M.G. Farquhar, San Diego, CA, USA
Integrin β1	-	Rabbit (Antisera)	1:100 (IF)	S. Johansson, Uppsala, Sweden
$p27^{KIP1}$	Clone 57	Mouse (M)	1:1000 (IB)	Transduction Laboratories
Phosphotyrosine	PY20 4G10 PT-66	Mouse (M) Mouse (M) Mouse (M)	1:1000 (IB) 1:1000 (IB) 1:100 (IF)	Transduction Laboratories Upstate Biotechnologies Sigma
Retinoblastoma protein	G3-245	Mouse (M)	1:1000 (IB)	Pharmingen
Smooth muscle α-actin	1A4	Mouse (M)	1:100 (IF) 1:1000 (IB)	Sigma
Vinculin	HVIN-1	Mouse (M)	1:400 (IF)	Sigma

M, monoclonal, P, polyclonal

RAT CAROTID BALLOON INJURY MODEL

In order to correlate in vitro findings regarding cyclin D1 expression and caveolae numbers, with SMC activation in vivo, the rat carotid balloon injury model was used.

This model was initially described and characterized by Clowes and coworkers [Clowes et al., 1983a; Clowes et al., 1983b]. Male Sprague Dawley (300-400 g) rats were anesthetized with Hypnorm and the distal left common carotid and external carotid arteries exposed by a midline neck incision. A 2F balloon catheter was introduced into the left common carotid artery through an arteriotomy in the external carotid artery. The balloon was then inflated and passed throughout the length of the left common carotid artery three times. The external carotid artery was ligated and the animals sacrificed after different time periods. Non-patent vessels at the time of sacrifice were excluded.

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The expression and distribution of cyclin D1 in the rat carotid artery after balloon injury was studied by immunohistochemistry. The right and left common carotid arteries were perfusion and immersion fixed in 4% formaldehyde, embedded in paraffin and sectioned. The sections were dewaxed in xylene and rehydrated to water through graded alcohols. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in 70% methanol. To unmask hidden epitopes, the slides were boiled in citrate buffer pH 6.0 for 10 minutes. Nonspecific binding was blocked with 1% goat serum in PBS. After overnight incubation with anti-cyclin D1, the slides were washed with TBS and immunodetection performed using the ABC method (Vector Laboratories) with diamino-benzidine (DAB) was as a chromogenic substrate. Methyl green was used to counterstain the cells.

RESULTS AND DISCUSSION

This thesis is based on results from our studies of integrin related-signaling pathways during the phenotypic modulation and activation of freshly isolated SMCs cultured on a substrate of FN. The results are presented in three main sections. The first section deals with changes in fine structure and cell cycle regulation during primary culture of SMCs on FN. Specific attention has been paid to the changes in expression of cell cycle regulatory proteins and caveolae, a sub-compartment of the plasma membrane that has been reported to be enriched in a large variety of signaling molecules (papers II and IV). In the second section, I present results showing the activation of integrin-related signaling molecules and their significance for the modification of SMC function in primary culture based on experiments using specific inhibitors (Papers I, III and V). These results increased our knowledge about the molecular pathways involved in the phenotypic modulation process and provided the necessary background for paper V, which is presented in the last section. Here, we have evaluated the effect of a clinically used drug, mevinolin (lovastatin) on the activation of SMCs cultured on a substrate of FN.

CHANGES IN SMC FINE STRUCTURE AND CELL CYCLE ENTRY AND PROGRESSION (PAPERS II AND IV)

Caveolae abundance is associated with phenotypic modulation

We have previously reported that the activation of freshly isolated SMCs on a substrate of FN is associated with characteristic changes in cellular morphology as judged by electron microscopy. This includes loss of myofilaments and development of an extensive ER and a large Golgi apparatus [Hedin and Thyberg, 1987]. However, the presence and distribution of caveolin and caveolae, which are small invaginations in the plasma membrane with key functions in signal transduction [Fielding and Fielding, 2000; Schlegel and Lisanti, 2001], had not been studied before during phenotypic modulation of SMCs. We, therefore analyzed the abundance of caveolae by electron microscopy and caveolin expression by immunoblotting in SMCs in primary culture (Paper II). As the cells underwent the transition from a contractile to a synthetic state when cultured either in the presence of serum or on a substrate of FN in serum-free medium, we observed a significant decrease in the number of caveolae at the plasma membrane. The abundance of caveolae was more related to the phenotype of the cells than on the time in culture, since even the few cells that were still in a contractile phenotype as determined by structural features after 6 days of culture had more caveolae compared to synthetic cells on the same day. Caveolae numbers also increased when cells grown in serum reached confluency and stopped to divide, suggesting an involvement in growth rate control. Since caveolin, the major coat protein of caveolae, is essential for their formation [Rothberg et al., 1992], we speculated that synthetic cells would have a decreased expression of caveolin

compared to contractile cells. However, the levels of caveolin-1 remained unchanged as SMCs were grown in primary culture. Moreover, immunofluorescence microscopy showed that caveolin shifted from the plasma membrane to the perinuclear cytoplasm over time. Internalization of caveolin and caveolin-containing vesicles has been observed in diverse processes such as in endothelial cells under oxidative stress [Aoki et al., 1999] and endocytosis of viral particles by CV-1 cells [Pelkmans et al., 2001]. If the 'Caveolae Signaling Hypothesis', which postulates that caveolae are a sub-compartment of the plasma membrane where signaling events occur (see Introduction; [Lisanti et al., 1994; Schlegel and Lisanti, 2001]) is correct, then the change in localization of caveolin could account for part of the differences in signaling and cellular response to similar stimuli between different cellular phenotypes. It is also possible that the signaling molecules, once activated, are detached from caveolae and that caveolin and other components of the membrane in caveolae at the same time are internalized. This could then explain why caveolae are fewer in number in synthetic than in contractile SMCs. In addition, the internalization of caveolin could be part of a receptor down regulation process characterizing many signaling systems. To relate our results regarding change in caveolae abundance and phenotype to the activation of SMCs in vivo, we used the rat carotid balloon injury model. As expected, caveolae were most abundant in medial SMCs in non-injured control arteries and distinctly lower in intimal SMCs one week after balloon injury. Thereafter, the number of caveolae in the SMCs in the neointima increased again until day 35, but were still significantly lower than in the non-injured artery. Thus, similar to our observations in culture, contractile cells in the artery had more caveolae than synthetic cells. In support of these findings, we subsequently found that caveolae numbers were higher in cells treated with drugs that inhibited the phenotypic modulation process (papers III and V). Together, these results suggest that caveolae abundancy is closely related to the phenotypic state of SMCs both in vivo and in vitro. Also in other cell systems, caveolae numbers have been shown to be higher in differentiated or non-transformed cells compared to non-differentiated or transformed cells [Engelman et al., 1997; Petersen et al., 1989] suggesting that the described phenomenon is not limited to SMCs.

Matrix-mediated cell cycle control

One of the most important functional consequences of the phenotypic modulation is to make SMCs competent to respond to growth factors and proliferate. This is illustrated by the observation that SMCs in primary culture do not synthesize DNA and proliferate in response to growth factor stimulation until the cells have acquired synthetic properties [Hedin and Thyberg, 1987]. In paper IV, we confirmed these observations by analyzing DNA content in cells grown on FN under serum-free conditions using flow cytometry. Over 94 % of the cells were in the G_0/G_1 phase of the cell cycle between day 0 and day 6. After serum stimulation, about 40 % of the cells entered the S-phase. In agreement with the increased proliferative capacity associated with the modulation process, a greater number of cells responded to growth

factor stimulation on day 4 than on day 2. In order to better understand the molecular basis for these findings, we analyzed if interactions with FN were associated with changes in cell cycle regulation. We chose to study the expression of cyclin D1 since the D-type cyclins are the first cyclins to be induced upon entry into the cell cycle [Sherr, 1993]. SMCs in the media of a non-injured artery are quiescent and reside in the G0 phase of the cell cycle. It was therefore not surprising that freshly isolated cells lacked cyclin D1. As early as on day 2 after adhesion to FN in the absence of serum, cyclin D1 was induced and the levels then increased up to day 6. Growth factor stimulation further increased cyclin D1 levels, suggesting that FN and growth factors cooperate in cyclin D1 induction. These findings are consistent with recent data in the literature [Assoian and Schwartz, 2001; Roovers et al., 1999; Weber et al., 1997] indicating that both growth factors and adhesion to the ECM are necessary for cell cycle progression in non-transformed cells. In contrast to these studies [Roovers et al., 1999; Weber et al., 1997], we observed cyclin D1 induction in the absence of exogenous growth factors. The reports cited above are based on results from experiments with passaged fibroblasts that were serum-starved for 48-72 hours and allowed to adhere and spread on a FN substrate for a couple of hours. In our experimental model, freshly isolated SMCs that are cultured on FN under serum-free conditions actively synthesize RNA and proteins, make new organelles, change their shape and form new FAs. This almost 'total overhaul' of the cells takes place over a period of 4 to 6 days. Thus, the mechanisms that regulate cyclin D1 induction may

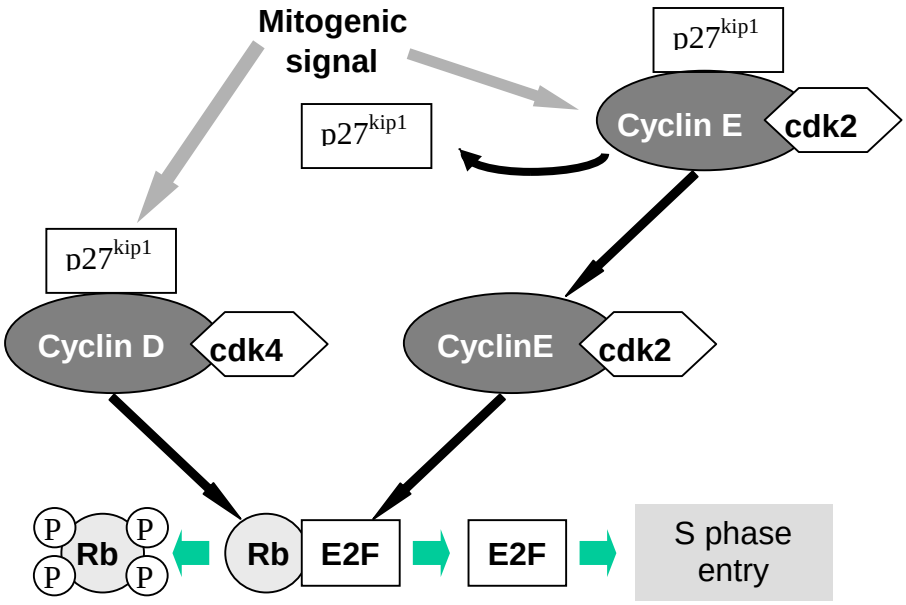

Figure 13: *Regulation of S-phase entry. Mitogenic signals promote the assembly of active cyclin D-cdk4 (or 6) complexes containing a CKI, $p27^{KIP1}$. Sequestration of $p27^{KIP1}$ facilitates the activation of the cyclin E-cdk2 complex. The cyclin D, E – cdk complexes phosphorylate Rb, which dissociates from E2F and allows this factor to activate genes required for S-phase entry.*

vary depending on the cell system studied and our results from a primary culture model may not be comparable to results from passaged cells. In fact, we have observed that cyclin D1 remains expressed at low levels in serum-starved passaged SMCs (Roy et al., unpublished data). As shown in Fig. 13, cyclin D1 and cyclin E bind cdk 4/6 and cdk 2, respectively, and the active kinase complexes regulate S-phase entry by hyperphosphorylating Rb [Sherr, 1993; Sherr and Roberts, 1999]. To detect if the induced cyclin D1 was active, we analyzed hyper-phosphorylated Rb by western blotting. Rb was not hyperphosphorylated in SMCs on FN indicating that the induced cyclin D1 was inactive and was not followed by cyclin D1-dependent kinase activity. Instead, Rb hyperphosphorylation and cyclin A expression, which is induced during the G_1/S phase transition [Sherr, 1993], required serum stimulation.

Cell-matrix interactions have been shown to promote cell cycle progression by down-regulating the CKI, $p27^{KIP1}$ [Fang et al., 1996; Koyama et al., 1996]. We therefore tested the hypothesis that the effect of FN on the ability of SMCs to respond to growth factor stimulation depended on decreased $p27^{KIP1}$ levels. However, this was not the case and SMCs grown on FN under serum-free conditions rather accumulated $p27^{KIP1}$. The activity of this CKI is mainly regulated by changes in protein stability [Poon et al., 1995] and degradation [Hengst and Reed, 1996; Morisaki et al., 1997; Pagano et al., 1995] and the observed accumulation in cells grown on FN is probably due to a block in the G1 phase rather than a FN-mediated increase in $p27^{KIP1}$mRNA levels [Lloyd et al., 1999]. The accumulation of $p27^{KIP1}$ could be due to a lack of cyclin E/cdk2-mediated degradation. This complex is probably inactive in SMCs in primary culture under serum-free conditions since no hyperphosphorylation of Rb was observed. However, in its active form, cyclin E/cdk2 has been shown to phosphorylate $p27^{KIP1}$, reducing the stability of this protein and thereby facilitating its degradation [Vlach et al., 1997].

The expression of cyclins E and A and the CKI's, $p27^{KIP1}$ and $p21^{CIP1}$ have been studied after arterial injury [Chen et al., 1997; Tanner et al., 2000; Tanner et al., 1998; Wei et al., 1997]. In general, the expression of these cyclins follows the pattern expected based on observations in other non-transformed proliferating cells i.e. the appearance of cyclin E followed by cyclin A. After vascular injury, an initial rise in $p27^{KIP1}$ and $p21^{CIP1}$ levels has been found, followed by a decrease during SMC proliferation in the injured artery. However, little is known about the expression and localization of cyclin D1 after arterial injury [Shigematsu et al., 2000]. Therefore, we studied the expression of cyclin D1 in the arterial wall after balloon injury of the rat carotid artery. Cyclin D1 was not detected in non-injured arteries but was induced in the arterial media 1 day after injury. The immunoreactivity for cyclin D1 then shifted to the neointima, studied 7 and 14 days after injury. The localization and expression of cyclin D1 correlated well to the areas of SMC activation and proliferation in the rat carotid artery [Pickering et al., 2000; Thyberg et al., 1995]. These results indicate that cyclin D1 is induced early after injury and is temporally and spatially expressed in the areas of SMC activation.

MATRIX-MEDIATED SIGNALING IN SMC ACTIVATION (PAPERS I, III, V)

After establishing that FN promotes phenotypic modulation and cell cycle entry in SMCs in primary culture, we wanted to investigate the mechanisms involved. Previously, it had been shown that soluble RGD peptides and antibodies against the rat β1 integrin subunit could prevent phenotypic modulation [Hedin et al., 1988], suggesting that this process was dependent on interactions between integrins, more specifically integrin α5β1 [Bottger et al., 1989; Hedin et al., 1989; Hedin et al., 1990], and the ECM. However, we were not able to study the significance of this integrin since functional rat-specific antibodies against integrin α5β1 were unavailable at the time. Instead, we directed our efforts towards cellular signaling pathways associated with integrins, cell spreading and cell cycle entry/progression.

Sustained activation of signaling molecules during phenotypic modulation.

Adhesion of various cell types, including SMCs, to ECM proteins is followed by a rapid increase in tyrosine phosphorylation [Guan et al., 1991; Kornberg et al., 1991; Schlaepfer and Hunter, 1998]. To investigate tyrosine phosphorylation in SMCs after adhesion and primary culture on ECM substrates, cell lysates were analyzed by immunoblotting using a monoclonal antibody against phosphotyrosine (Paper I). In cells cultured on FN, the most prominent tyrosine phosphorylated proteins were found around 125 kD, 90 kD and 75 kD in molecular weight. The ~125 kD protein was identified as FAK (Paper I). Paxillin, a 68 kD adaptor protein primarily localized in FAs [Turner et al., 1990], was probably the tyrosine phosphorylated protein migrating around the 75 kD molecular weight marker. In contrast to passaged SMCs (Paper I) and fibroblasts [Burridge et al., 1992; Lin et al., 1997], where FN-mediated FAK phosphorylation reaches a maximum 20 – 30 minutes after adhesion and then stays at this level for several hours, FAK phosphorylation in SMCs in primary culture on a substrate of FN was detected after 3-5 hours after adhesion and was maintained at constant levels for up to 96 hours. This extended activation of FAK probably reflects the time needed for these cells to adhere to the ECM, go through a progressive cell spreading process (which requires the formation of new plasma membrane, cytoskeletal components and intracellular organelles) and change their phenotype. The sustained activation of FAK observed under these conditions could also be due to the continuous formation of new ECM-integrin interactions mediated by the endogenous formation of a new pericellular matrix. Immunofluorescence microscopy showed that the phosphotyrosine staining was distributed in a FA-like pattern, suggesting that phosphorylated FAK and other tyrosine phosphorylated proteins were mainly situated in FAs (Paper I). One of the downstream targets of FAK is Ras, a 21 kD protein located upstream of ERK (see Introduction, Fig. 9; [Marshall, 1995]). ERK activation has been studied extensively in numerous cell types, especially after the introduction of phosphospecific antibodies that detect active ERK, and the introduction of specific ERK inhibitors such as PD98059 [Alessi et al., 1995; Dudley et al., 1995] and U0126

[DeSilva et al., 1998; Duncia et al., 1998; Favata et al., 1998]. In the SMCs, ERK1/2 was found to be activated during isolation of the cells by collagenase digestion. However, the activity did not disappear after adhesion and was sustained for up to 6 days in culture on FN under serum-free conditions (paper III). This is in contrast to passaged SMCs, in which ERK is only transiently activated after adhesion to FN and no active ERK could be detected after 4 hours (Paper III). However, the levels of ERK1/2 activation during primary culture on FN were not maximal and growth factor stimulation further increased these levels.

So what was the real significance of these signaling molecules in the process of SMC activation during primary culture on FN? One may use different approaches to test this. First, overexpression studies with constitutively active or inactive molecules can be performed. The other way, which we chose because of the difficulties associated with stable transfection of primary cells, is to use specific inhibitors. The main disadvantage of this method is that the drugs may turn out not to be as specific as they were originally reported to be. Both genistein and PD98059 inhibited phenotypic modulation of the SMCs, suggesting that integrin-linked tyrosine kinase activity and sustained ERK activation were indispensable for this process. Genistein is a general tyrosine kinase inhibitor [Akiyama et al., 1987; O'Dell et al., 1991] and its effects on SMC phenotype may be due to inhibition of tyrosine kinases other than FAK. PD98059 is a specific MEK1 inhibitor, which prevents the phosphorylation of ERK1/2 by MEK1 [Alessi et al., 1995; Dudley et al., 1995]. Unlike U0126, it does not have an effect on active ERK already present in the cells but rather inhibits de novo activation of ERK [DeSilva et al., 1998; Duncia et al., 1998; Favata et al., 1998]. The fact that PD98059 efficiently blocked phenotypic modulation implies that the phenotypic modulation and activation on FN is dependent on a continuous activation of ERK1/2 during culture rather than an effect of ERK1/2 activation already during collagenase digestion. At this stage we were tempted to think that the effect of FN on phenotypic modulation was mediated through activation of FAK, which in turn can activate ERK [Aplin et al., 1998]. On the other hand, FAK is not essential for ERK activation since FN has been shown to activate ERK in FAK deficient fibroblasts [Sieg et al., 1998]. The inhibition of phenotypic modulation by PD98059 was accompanied by an inhibition of cyclin D1 (Paper III), which is in agreement with the notion that sustained ERK1/2 activation is necessary for cyclin D1 induction [Roovers and Assoian, 2000]. Therefore, we concluded that the sustained sub-maximal levels of phosphorylated ERK1/2 found in primary SMCs played a significant role in the change of phenotype and cell cycle entry.

As previously mentioned, not only the change in phenotype but also cell spreading and FA formation were inhibited in SMCs cultured on laminin or on FN in the presence of drugs such as PD98059 and genistein (papers I and III; [Hedin et al., 1988]). Work pioneered by Ridley and Hall established that the small GTP-binding protein RhoA is essential for FA assembly [Hall, 1998; Ridley and Hall, 1992; Ridley et al., 1992]. In order to further elucidate the mechanisms involved in the association between cell

spreading, FA formation, and SMC phenotype, we went on to analyze the role of Rho in this context (Paper V). C3 exoenzyme is a *Clostridium botulinum* toxin that specifically inactivates Rho by ADP ribosylation at Asn41 [Aktories, 1997]. It has been demonstrated that C3-catalyzed ribosylation of Rho is 100-400 times more efficient than that of other proteins of the Rho-family such as Rac and Cdc42 [Ridley and Hall, 1992; Ridley et al., 1992]. C3 exoenzyme treatment prevented cell spreading and the cells remained spindle shaped. Electron microscopy confirmed that C3 exoenzyme treatment also inhibited the internal structural reorganization of the cells, indicating that Rho activity is necessary for phenotypic modulation of SMCs. Further evidence for the role of Rho in this process was obtained in studies with statins (see below).

Laminin-mediated signaling

Freshly isolated SMCs cultured on a substrate of laminin retain a contractile phenotype during the first few days in culture. However, under these conditions, the cells start to synthesize FN on their own, thereby creating a FN-containing pericellular matrix that in turn promotes cell spreading and phenotypic modulation. After 5 to 6 days in culture, most of the cells are thus in a synthetic state. The interaction between the endogenous FN and SMCs can be blocked by adding soluble RGD peptides or antibodies against the β1 integrin subunit to the culture medium and thereby prevent the modulation process [Hedin et al., 1988]. In order to identify differences in signaling mechanisms between cells cultured on laminin and FN substrates, we also analyzed the activation of ERK1/2 and FAK in SMCs cultured on laminin in primary culture. Cells cultured on laminin remained spindle shaped and formed few FAs as identified by vinculin staining. In contrast to cells seeded on FN, immunofluorescence microscopy and Western blotting showed low levels of phosphotyrosine and phosphorlyated FAK in SMCs cultured on laminin. ERK1/2 activation was also inhibited when cells grown on laminin were incubated with RGD peptides to block the interaction with FN. In summary, the signaling pathways involved in SMC phenotypic modulation on a substrate of FN were not activated when cells were cultured on laminin, supporting the assumption that this processs is intimately coupled to the ability of the ECM to promote FA formation and cell spreading and to activate associated signaling pathways. However, experiments using freshly isolated SMCs were difficult to perform on a substrate of laminin. Cells adhered less efficiently to laminin than to FN and the adhesion efficiencies varied depending on the batch of laminin used (~30-35% as compared to ~75% with FN). Preliminary results from our laboratory suggest that SMCs adhere more efficiently to recombinant laminin-11 and laminin-10 than to laminin-1 (Lundmark et al., unpublished observations), suggesting that further studies should be done to confirm whether or not the ability to support the expression of a more differentiated phenotype is shared by different laminins.

THE HMG-COA REDUCTASE INHIBITOR MEVINOLIN BLOCKS PHENOTYPIC MODULATION (PAPER V)

Statins or HMG-CoA reductase inhibitors are widely used as drugs to treat hypercholesteremia and have been shown to reduce cardiovascular related morbidity and mortality in primary and secondary prevention trials [Maron et al., 2000]. However, it has been argued that the benefits observed cannot entirely be related to their effects on blood-lipid levels, suggesting that other mechanisms may be involved [Maron et al., 2000; Massy et al., 1996; Rosenson and Tangney, 1998; Vaughan et al., 1996]. We were interested in the effects of statins on phenotypic modulation, since our earlier results regarding caveolae and phenotype raised the possibility that drugs affecting cholesterol transport or metabolism may affect SMC phenotype.

In paper V, we tested the commercially available lipophilic statin mevinolin, also known as lovastatin, in cultures of freshly isolated SMCs cultured on FN under serum-free conditions. At a concentration of 5 μM, mevinolin had a profound effect on cell spreading and cellular fine structure without affecting cell adhesion to FN. In a similar manner as genistein and PD98059 (paper I and III), mevinolin prevented cell spreading

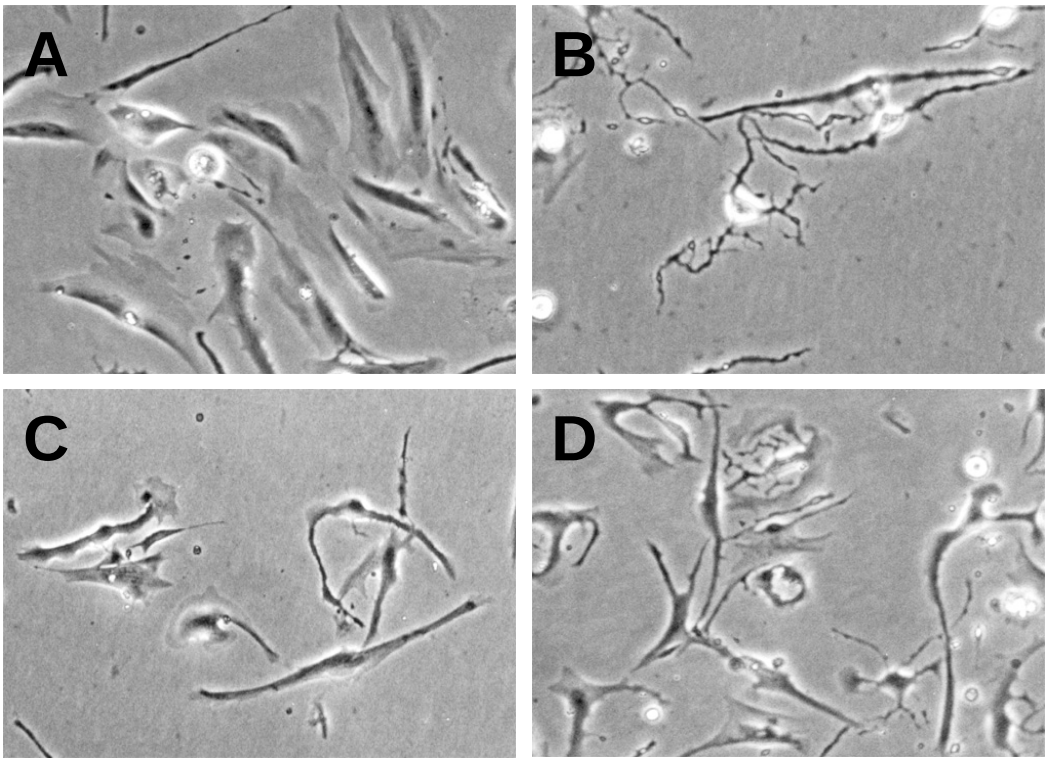

Figure 14: *Reversal of the effect of mevinolin on cell spreading of SMCs in primary culture on FN. (A) SMCs after 3 days of culture on FN in serum-free medium. (B) Cultures after 3 days on FN in serum-free medium with 5 μM mevinolin. Cell spreading 1 hr (C) and 3 hrs (D) after removal of mevinolin and addition of 10% FCS.*

and the cells remained spindle-shaped throughout the culture period. Vinculin and SM α-actin staining showed that mevinolin blocked the formation of FAs and reorganization of the actin cytoskeleton into stress fibers. Removal of mevinolin after 4 days was followed by a rapid spreading and the cells attained a flattened shape after 3 hours showing that the effects on cell spreading were reversible and not due to a general cytotoxic effect of the drug (Fig. 14). Electron microscopic analysis further showed that the mevinolin-treated cells were retained in a contractile phenotype, i.e. the cytoplasm was filled with myofilaments and the ER and Golgi apparatus were small in size. Furthermore, caveolae numbers were higher in mevinolin-treated than in control cells. The levels of SM α-actin, which normally decrease when SMCs are established in culture and modulate from a contractile to a synthetic state [Blank and Owens, 1990], were also higher in mevinolin-treated cells than in control cells.

So what was the mechanism of action by which mevinolin prevented phenotypic modulation?

Statins decrease plasma cholesterol levels by inhibiting HMG-CoA reductase, the enzyme that catalyzes the rate limiting step in cholesterol biosynthesis i.e. the conversion of HMG-CoA to mevalonate (Fig. 15; [Endo et al., 1977]). In addition, FPP and GGPP are involved in the post-translational modification of various proteins and serve as lipid attachments to plasma membranes. These proteins include the small GTP-binding protein Ras and the Rho-family proteins such as Rho, Rac, Cdc42, Rap1, Ral and Rab [Bar-Sagi and Hall, 2000]. Our initial idea that statins affected

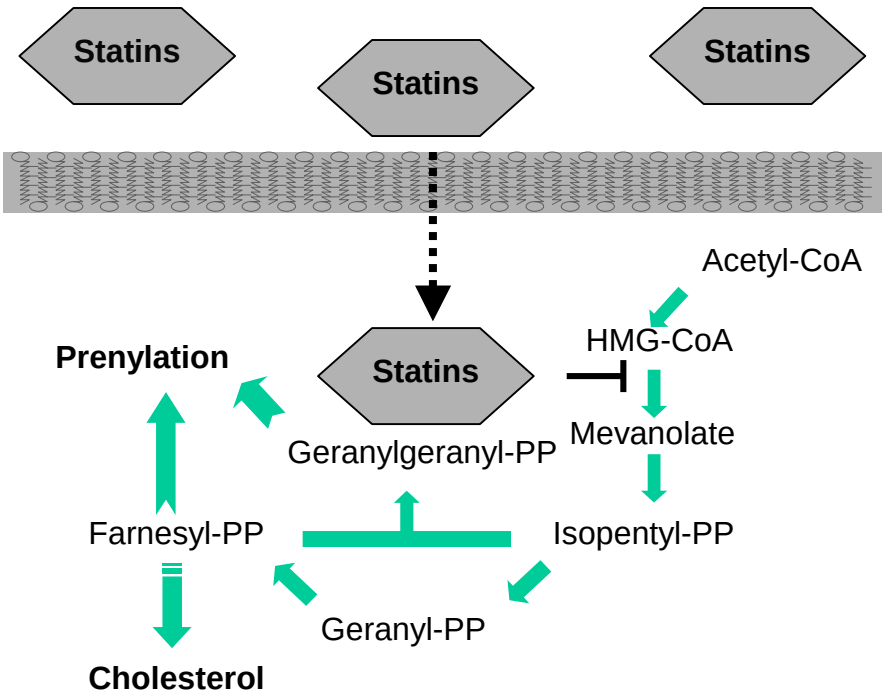

Figure 15: *Effects of statins on isoprenoid and cholesterol synthesis.*

SMC phenotype through an effect on cholesterol levels turned out to be incorrect since addition of mevalonate but not cholesterol could reverse the effects of mevinolin. This finding suggested that metabolites derived from mevalonate other than cholesterol were involved. Accordingly, addition of GGPP but not FPP was found to neutralize the effects of mevinolin, indicating that geranylgeranylation is necessary for phenotypic modulation of SMCs. Whereas Ras is farnesylated, Rho, Rac and Cdc 42 are geranylgeranylated [Seabra, 1998]. Since GGPP counteracted the effects of mevinolin, Rho, Rac and Cdc42 rather than active Ras seemedto be critical for phenotypic modulation. Indeed statins have been shown to affect other processes such as SMC migration and proliferation, and endothelial cell endothelial nitric oxide synthase expression by inhibiting the geranylgeranylation of Rho [Laufs and Liao, 2000]. This is a likely mechanism through which mevinolin inhibits phenotypic modulation since mevinolin-treated cells lack FAs, the formation of which requires active Rho [Ridley and Hall, 1992]. However, our results cannot exclude the possibility that mevinolin may affect SMC phenotype by inhibition of other geranylgeranlyated proteins than Rho. We also investigated whether the effect of mevinolin was due to inhibition of ERK1/2, since treatment with the MEK1 inhibitor, PD98059 (paper III) had been found to interfere with phenotypic modulation in a similar manner as the Rho inhibitor, C3 exoenzyme. We did not observe any significant differences in the active ERK1/2 levels in control and mevinolin-treated cells. Most likely, sustained levels of active ERK1/2 are necessary but not sufficient for the phenotypic modulation of SMCs. In contrast, FAK phosphorylation was inhibited by mevinolin treatment, which is in agreement with the idea that Rho activity is involved in the activation of FAK. Clark and co-workers have shown that when fibroblasts adhere to FN, FAs form in the absence of significant FAK phosphorylation, and subsequent formation of FAs and further FAK phosphorylation occur in a Rho-dependent manner [Clark et al., 1998]. In addition to a block in phenotypic modulation, mevinolin treatment inhibited cyclin D1 induction, which could be rescued by adding mevanolate and GGPP. Previously, we had observed that cyclin D1 expression was dependent on ERK activation (see above, paper III). It was, therefore, surprising that mevinolin-treated cells had sustained levels of ERK1/2 phosphorylation since this pathway has previously been proposed to be critical for cyclin D1 induction [Roovers and Assoian, 2000]. On the other hand, Danen and coworkers could inhibit cyclin D1 in growth factor stimulated NIH3T3 cells by C3 exoenzyme treatment without affecting the levels of phosphorylated ERK1/2, suggesting that Rho activity may also be necessary for cyclin D1 induction [Danen et al., 2000]. The interplay between ERK1/2, Rho, FAK and cyclin D1 are discussed in more detail below (See General Discussion)

As with passaged SMCs, mevinolin inhibited proliferation of SMCs in primary culture [Choi et al., 1995; Corsini et al., 1993; Corsini et al., 1996; Munro et al., 1994]. This property seems to be shared by most of the different statins and interestingly, GGPP rather than FPP can reverse the effects of mevinolin. This is in contrast to results in neoplastic cell lines where FPP rather than GGPP has been shown to reverse the

effects of statins on cell proliferation, suggesting that Ras rather than Rho is essential for cell proliferation in cancer cells while the opposite holds for non-neoplastic cells such as SMCs [Laufs and Liao, 2000]. To support this idea, we studied the effects of mevinolin on serum-stimulated DNA synthesis and ERK phosphorylation. Although mevinolin could strongly inhibit serum-induced DNA synthesis (Fig. 16), we observed very little changes in the levels of ERK1/2 phosphorylation (unpublished observation). Recently, it was shown that another statin, simvastatin inhibited DNA synthesis in passaged SMCs but similarly to mevinolin had little effect on growth factor stimulated ERK1/2 phosphorylation. In addition, it was demonstrated that simvastatin prevented GTP- loading of both RhoA and Rac1. These results indicate a critical role of the Rho-family proteins in growth factor stimulated SMC proliferation [Negre-Aminou et al., 2001].

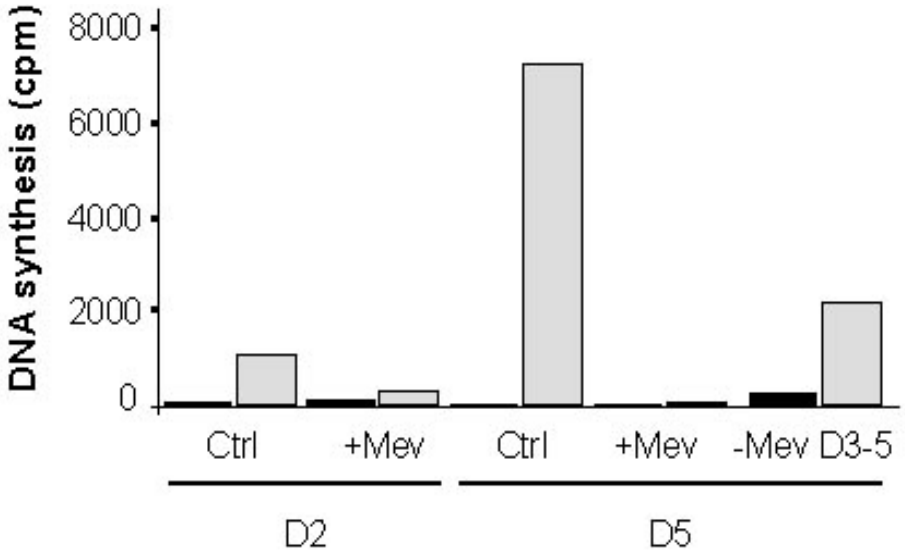

Figure 16: *DNA-synthesis in SMCs after 2- (D2) and 5 (D5) days of primary culture on FN in the presence (Mev) or absence (Ctrl) of 5 μM mevinolin. After 2 and 5 days, the cells were stimulated with (grey bars) or without (black bars) 10% FCS for 24 hrs and DNA synthesis estimated by ^{3}H-thymidine labeling. Note response in cultures where mevinolin was removed after 3 days (-Mev D3-5).*

In addition to effects on phenotypic modulation and serum-stimulated cell proliferation, lipophilic statins have been shown to induce apoptosis of passaged SMCs [Guijarro et al., 1998]. Accordingly, mevinolin induced an increased level of apoptosis (25% vs 5% in control cultures) in SMCs in primary culture on a substrate of FN as judged by TUNEL staining and electron microscopy (Paper V). However the pathways involved in phenotypic modulation and apoptosis may differ since both FPP and GGPP could reverse the effect of statin treatment.

GENERAL DISCUSSION

The development of intimal hyperplasia after vascular injury is a complex process. Knowledge from humans and from animal models indicates that one of the initial events is the transition of SMCs from a contractile to a synthetic phenotype [Hedin and Clowes, 1996]. Only after undergoing this modification, can SMCs migrate, proliferate and deposit ECM, key events in the formation of intimal hyperplasia. Numerous clinical trials have been performed to prevent this process by targeting SMC proliferation only, but without success [Mak and Topol, 1997]. A multifaceted approach to treat the disease based on fundamental aspects of SMC function should be more relevant and successful. ECM composition and growth factor expression regulate SMC function during atherogenesis and after vascular injury. To study the role of the ECM in phenotypic modulation, we have used a well-established in vitro system [Thyberg, 1996a]. Enzymatic digestion of rat aortas produces a pure population of SMCs in a phenotypic state resembling that existing in the normal media. These cells can then be seeded on ECM components and grown in serum-free medium under defined conditions. Explant and passaged cultures are unsuitable, since the cells in these cases have already shifted from a contractile to a synthetic state. A major advantage of the system is that it enables us to differentiate between ECM- and growth factor-mediated intracellular signaling and cell cycle events.

INTRACELLULAR SIGNALING PATHWAYS INVOLVED IN PHENOTYPIC MODULATION.

Freshly isolated SMCs change their phenotype when cultured on a substrate of FN under serum-free conditions. Initially the cells are spindle shaped and then start to spread, form focal adhesions and stress fibers, and attain a flattened shape. Fine structural analysis shows that the cells lose much of their myofilaments and that the cytoplasm becomes filled with ER and Golgi complex. This process is more or less complete around the fourth day after seeding [Thyberg, 1996a]. This thesis demonstrates that FN-mediated phenotypic modulation is dependent on integrin-linked tyrosine kinase, ERK1/2 and Rho activity. Inhibition of either of these pathways with the pharmacological agents genistein, PD98059 and C3 exoenzyme, blocked phenotypic modulation, as determined by fine structural analysis. As a general part of their effects, these drugs inhibited focal adhesion formation, cytoskeletal reorganization, and cell spreading.

Recently the Rho proteins have been shown to be key regulators of the actin cytoskeleton and actin filament-dependent signaling. This could explain the inhibitory effect of the specific Rho inhibitor, C3 exoenzyme. Genistein is a general tyrosine kinase inhibitor and its effects were probably mediated via interference with protein tyrosine phosphorylation downstream of integrin activation [Akiyama et al., 1987; O'Dell et al., 1991]. The reason why inactivation of ERK1/2 blocked phenotypic

modulation is not obvious. A possible mechanism could be via the down regulation of integrin expression as has been shown in osteoblasts [Lai et al., 2001]. Furthermore, it has been shown that blocking of ERK1/2 phosphorylation inhibits carbamoyl phosphate synthetase, the enzyme that catalyzes the rate-limiting step in the de novo syntheis of pyrimidine nucleotides [Graves et al., 2000]. Via this mechanism, PD98059 may prevent the necessary RNA and protein synthesis required for phenotypic modulation. Previous work, has shown that wortmannin, a PI-3 kinase inhibitor and tyrphostin A9, a PDGF-receptor tyrosine kinase inhibitor, did not prevent cell spreading or phenotypic modulation but affected SMC proliferation [Thyberg, 1998b]. Thus, the mechanisms involved in phenotypic modulation and SMC proliferation in primary culture do differ.

FN-MEDIATED CELL CYCLE PROGRESSION IN PRIMARY CULTURE

FN-mediated phenotypic modulation of SMCs in primary culture results in an increased ability to respond to growth factor stimulation [Thyberg, 1996a]. Objectively, this is reflected by increased thymidine incorporation and increased DNA content as determined by flow cytometry (paper IV; [Hedin and Thyberg, 1987]). Interestingly, SMCs in primary culture on a substrate of FN were observed to require only 30-60 minutes of exposure to PDGF-BB while passaged cells required 4-8 hours to reach maximum levels of DNA synthesis [Thyberg, 1996b]**.** Hence the change in differentiated state that occurs during the first days of cultivation clearly ‘primes’ the cells to enter the cell cycle. In support of this notion, it was here observed that cyclin D1 is induced during phenotypic modulation of SMCs cultured on FN under serum-free conditions, indicating that the cells entered the G1 phase. Furthermore, cell cycle entry was coupled to cytoskeletal reorganization and dependent on integrin-linked tyrosine kinase and ERK1/2 activity. The change in cell shape and induction of cyclin D1 may be a mechanism through which integrins facilitate the ability of SMCs to respond to growth factors and progress to the S-phase.

An emerging theme in anchorage-dependent cell cycle progression is the cooperation between growth factors and integrins to activate the signaling pathways involved [Roovers and Assoian, 2000]. In work done on passaged fibroblasts, cyclin D1 was shown to rely on sustained ERK1/2 activation, which required both adhesion to a FN matrix and growth factor stimulation. The effect of the FN matrix was mediated via the phosphorylation of FAK or via other non-receptor tyrosine kinases or adaptor proteins such as Fyn or Shc. In our experiments, FAK phosphorylation was preceeded by ERK activation (Paper I and III). Therefore, we conclude that ERK1/2 activation was dependent on the phosphorylation of other tyrosine kinases than FAK. The role of cytoskeletal reorganization in cyclin D1 induction has been attributed to the activation of the Rho proteins. For example, Danen and coworkers observed that cyclin D1 induction in fibroblasts cultured on FN could be blocked by treatment with the C3 exoenzyme. On the other hand, laminin or collagen did not support cytoskeletal reorganization or Rho activation and the cells lacked cyclin D1 [Assoian and

Schwartz, 2001; Danen et al., 2000; Huang and Ingber, 1999; Roovers and Assoian, 2000]. Little is known about how integrins activate Rho. It has been suggested that integrins might directly activate GEFs or act further downstream and regulate the coupling of GTP-bound Rho proteins to their targets [Assoian and Schwartz, 2001; del Pozo et al., 2000; Sastry and Burridge, 2000].

RHO AS A PHARMACOLOGICAL TARGET

Work in this thesis demonstrates that Rho activation through cytoskeletal reorganization is critical for the activation of SMCs and cell cycle entry. Statins are well tolerated clinically used lipid-lowering drugs that have been shown to function in vitro through the inhibition of Rho [van Nieuw Amerongen and van Hinsbergh, 2001]. We therefore tested the effect of a lipophilic statin, mevinolin, on phenotypic modulation of SMCs in primary culture. Besides a strong inhibitory effect on phenotypic modulation as judged morphologically, mevinolin also blocked cyclin D1 induction and FAK phosphorylation, but not ERK1/2 phosphorylation. In consistence with the literature, mevinolin further decreased mitogen-induced DNA synthesis and promoted apoptosis in SMCs [Guijarro et al., 1998; Laufs and Liao, 2000]. These results suggest that statins can potentially target multiple aspects of SMC function in vascular diseases.

Statins block the isoprenylation of Rho proteins as a side effect of their inhibition of the HMG-CoA reductase enzyme [Goldstein and Brown, 1990]. This mechanism was supported by our observations, in which mevalonate and GGPP could reverse the effects of mevinolin but not cholesterol or FPP. In addition to their effects on SMCs, statins also lower blood-lipid levels, reduce inflammation and endothelial permeability, and increase fibrinolytic activity [Maron et al., 2000; van Nieuw Amerongen and van Hinsbergh, 2001]. Despite the promising nature of these results, clinical trials using statins to prevent restenosis have not shown convincing effects [Serruys et al., 1999; Weintraub et al., 1994]. One possible explanation of the negative results is that sufficient blood concentrations of the statins were not achieved. The principal action of statins is the inhibition of cholesterol synthesis in the liver, where most of the drug is metabolized. A local delivery method where statins are directly applied to the vascular wall at the time of injury could be a way to overcome this problem.

A SIMPLISTIC APPROACH TO SIGNALING IN THE CONTROL OF SMC PHENOTYPE AND CELL CYCLE PROGRESSION

The process of phenotypic modulation is indeed a complex one. Based on the results presented in this thesis, I propose that the ECM regulates phenotypic modulation and cell cycle entry via two linear pathways (Fig. 17). This is surely an over-simplification and multiple feedback mechanisms probably exist, as is usually the case in biological systems.

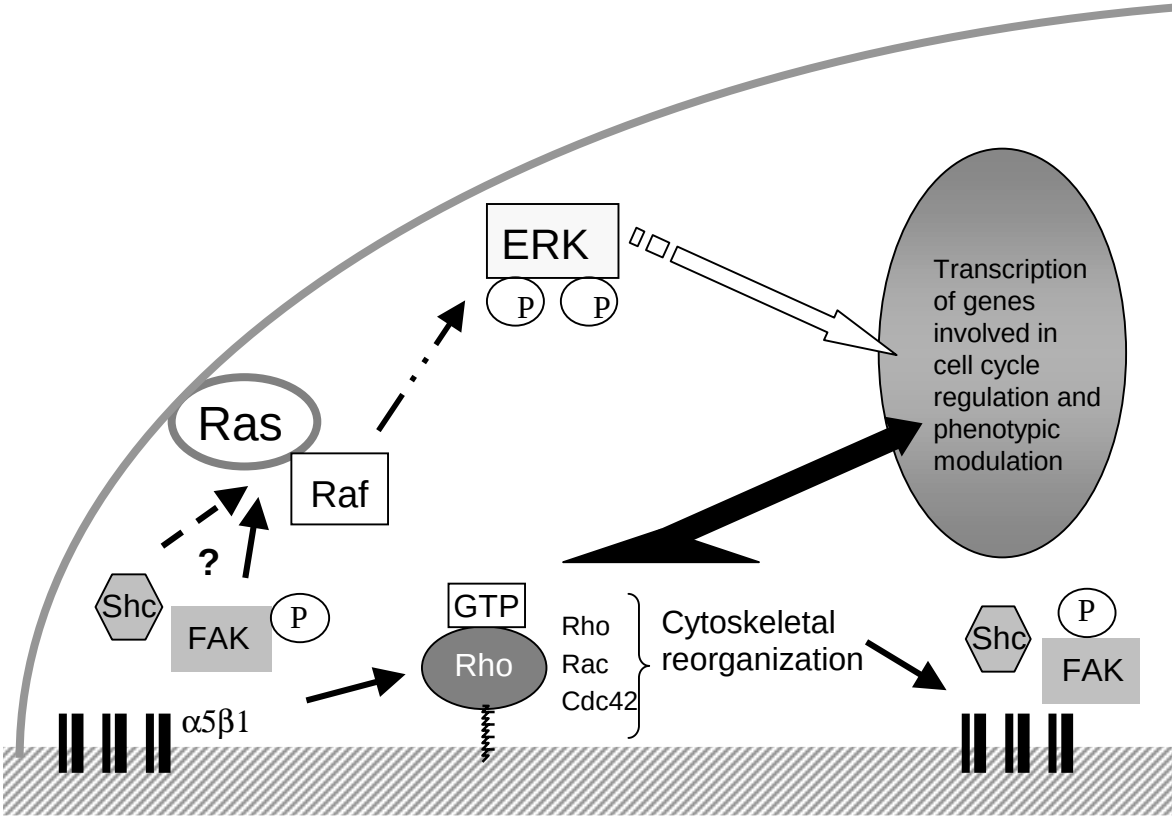

Fig. 17: *Schematic illustration of the intracellular signaling pathways involved in FN-mediated phenotypic modulation and cell cycle entry of SMCs. For details, see text.*

Binding of the α5β1 integrin to FN activates downstream tyrosine phosphorylation events including FAK phosphorylation. Thereafter the pathways diverge. One pathway leads to the activation of the Rho proteins, Rac, Cdc42 and Rho. This results in cell spreading, formation of FAs and stress fiber reorganization. The cell spreading process in turn facilitates the binding of new integrins to the FN matrix, creating a positive feedback effect on the system. The other pathway activates ERK1/2. This occurs either via activation of FAK or via other signaling molecules that recruit a MAPK kinase kinase such as Raf to the plasma membrane. Activated Raf then through a sequential phosphorylation cascade activates ERK1/2. These kinases are finally translocated into the nucleus where they affect transcription factors including Elk-1 and AP-1. The two pathways converge at a point downstream of ERK1/2 and Rho activation to induce cyclin D1 and phenotypic modulation.

WHERE DO WE GO FROM HERE?

The quest for finding the mechanisms that regulate the differentiated properties and function of SMCs has certainly not ended. We are probably just at the tip of the iceberg, surfing over the few pathways that are today known to cell biology. Furthermore, the characterization of the ECM in the vascular wall is not complete. Specifically, the composition of the BM surrounding SMCs and its role in signaling

has not been determined. Much more also remains to be learnt about the clustering and interaction of different signaling molecules in cellular membranes and the role of specialized membrane domains such as caveolae in this context. Improved knowledge of general cell biological issues of this type will help us to understand the functions of the cells in the vascular system and to develop new methods for treatment of vascular disease.

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