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Abstract

During migration, smooth muscle cells (SMC) modulate their interactions with surrounding matrix and cells. The goal of this study was to examine the lateral adhesion N-cadherin complex and how it is modulated as SMCs assume a migratory phenotype during proliferative responses to injury. N-cadherin was found localized at the basolateral extensions of contacting SMCs. Inhibition of N-cadherin complexes with blocking antibodies reduced the ability of SMCs to migrate in vitro. Morphologically, cells had shorter, blunted processes into the wound area that translated into significantly reduced wound edge growth at 24 and 48 hours after wounding. In vivo, western analysis and immunofluorescent staining showed that neointimal SMCs in rat carotid arteries expressed increased levels of N-cadherin, β-catenin and plakoglobin at 1 and 3 weeks and decreased levels at 8 weeks after balloon catheter injury. These results suggest that cell-cell contact mediated by N-cadherin is involved in the migration of SMCs.
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CHAPTER 1

Background Review
1.1 Introduction

Smooth muscle cell migration and proliferation are key contributors to intimal thickening observed in pathological conditions such as atherosclerosis and in the development of restenosis after bypass graft surgery, endarterectomy and percutaneous transluminal angioplasty. Migration of smooth muscle cells is preceded by a transition from a contractile, quiescent state to a proliferating, synthetic phenotype. As smooth muscle cells undergo this phenotypic alteration and migrate, cell-matrix and cell-cell contacts are dynamically regulated through the coordination of adhesion and de-adhesion events that enable cell movement. Cell-cell adhesion in vascular smooth muscle cells is mediated in part by cadherin molecules. These adhesion complexes have not been characterized in vascular smooth muscle cells nor has their regulation been described as the cell undergoes phenotypic alterations, proliferates and migrates. This literature review summarizes the cadherin family of adhesion molecules, their function and distribution. Emphasis will be placed on one member, N-cadherin, because it is the principle cadherin expressed in smooth muscle cells. Neointimal thickening will be reviewed in terms of causative factors, mechanisms involved and cell types present. Lastly, smooth muscle cells will be described with focus on some of the factors and molecules that are involved in the processes of phenotypic modulation and migration.
1.2 Cadherins

1.2.1 Structure and Function

Cadherins belong to a family of glycoproteins that mediate calcium dependent cell-cell adhesion. They are involved in homophilic interactions that are important in both the developing and adult organism. Cadherins are temporally and spatially regulated during normal embryonic development and contribute to key processes such as cell migration, cell recognition and sorting involved in neurulation, gastrulation, somitogenesis and cardiogenesis (Hatta et al., 1987; Hatta and Takeichi, 1986; Ong et al., 1998). Ectopic or altered expression of cadherins leads to disrupted development (Radice et al., 1997; Larue et al., 1994). In addition to their important and varied roles in embryogenesis, cadherins also regulate apoptosis, maintain tissue morphology and cell differentiation and establish cell polarity (Makrigiannakis et al., 1999; Islam et al., 1996; Ong et al., 1998).

Cadherins can be divided into 6 gene families based on their structures: classical type I, classical type II, cadherins found in desmosomes, cadherins with a very short or no cytoplasmic domain, protocadherins and distantly related gene products including Drosophila fat tumor suppressor gene, the dachsous gene, and the ret-protooncogene (Redies, 1995). The members of the classical cadherin subfamily, which includes N, P, R, B and E-cadherins are synthesized as precursor polypeptides and intracellular processing activates their adhesive properties (Aberle et al., 1996).

Cadhesion molecules exist as single chained transmembrane glycoproteins containing five major repeats that form four calcium binding pockets. In order for the cadherin molecule to become functional it is postulated that calcium binds in these pockets and organizes the cadherin into a rod like structure enabling it to form parallel cis dimers with adjacent cadherin molecules (Steinberg et al., 1999). Cis cadherin dimers of the classical type I family interact via the N-terminus adhesive domain composed of the amino acid sequence histidine, alanine and valine (HAV) (Takeichi, 1991; Blaschuk et al., 1990; Noe et al., 1999). This adhesive domain binds the N-terminus of another cadherin dimer on the opposing membrane forming a trans-dimer (Vleminckx and Kemler, 1999). These dimers are generally organized into specialized regions on the
plasma membrane called adherens junctions. Single amino acid mutations that are predicted to affect the formation of the cis-dimer impair cadherin functionality, indicating that the cis dimer arrangement supports formation of functional trans-dimers (Tamura et al., 1988).

Stabilization of the adherens junction involves the interaction of the cadherin cytoplasmic domain with the actin cytoskeleton through catenin proteins (Figure 1.1). The catenins consist of four known proteins, three of which are structurally similar and belong to the armadillo family. \( \beta \)-catenin (92kd), plakoglobin (82kd) and p120 (120kd) each contain a central armadillo domain consisting of 12-13 copies of a degenerate 42 amino acid motif originally described in the drosophila (Aberle et al., 1996). \( \beta \)-catenin and plakoglobin bind directly to cadherins via their armadillo repeats in a mutually exclusive fashion, giving rise to two distinct cadherin-catenin complexes. p120 binds directly to juxtamembrane domains of classical cadherins simultaneously with \( \beta \)-catenin or plakoglobin at non-overlapping regions and it appears to modulate adhesion in a manner that is independent of the other catenins (Provost and Rimm, 1999). \( \alpha \)-catenin (102kd), which has 3 regions of sequence homology with vinculin, binds via its amino domain to either plakoglobin or \( \beta \)-catenin, linking the complex to the actin filaments and promoting junction stabilization. The central domain serves as a binding site for actin-associated proteins \( \alpha \)-actinin and vinculin while its carboxy terminal portion interacts with another actin associated protein, ZO-1, and with F-actin and vinculin (Troyanovsky, 1999; Provost and Rimm, 1999). The association of catenins with the cytoplasmic domain of cadherins is believed to be important for the clustering of the receptors at sites of cell-cell contacts, providing strength to adhesion (Kemler, 1993).

1.2.2 Expression and Distribution in Epithelial-like cells

Cadherin expression is tissue specific; however, a single cell type often expresses more than one type of cadherin with or without the same distribution and function. Cadherins were originally identified in epithelial cells. These cells primarily express E-cadherin alone or in combination with P-cadherin. In normal epithelial cells, these
Figure 1.1: Cadherin adhesion dimer at the cell surface. Schematic includes some of the protein interactions within the adherens junction complex.
cadherins are localized at the basolateral surface of the cell within adherens junctions where they promote stable adhesion between cells. Formation of these intercellular junctions is required for the assembly of cells into multiple layers and is critical for the maintenance of cytoskeletal organization and epithelial polarization (Larue et al., 1994; Lewis et al., 1994; Rodriguez-Boulan and Nelson, 1989; Behrens et al., 1993). When epithelial cells become cancerous, increased levels of N-cadherin are expressed within the cell with or without a loss of E-cadherin. The expression of N-cadherin correlates with a change in the morphology of the cell from polarized epithelial to fibroblastic (Islam et al., 1996). The function of E-cadherin to promote and maintain an epithelial morphology is also demonstrated in cells that normally do not express E-cadherin. Introduction of E-cadherin in retinal pigment cells caused alterations in their polarity and promoted the assembly of desmosomes, a characteristic of epithelial cells (Marrs et al., 1995).

Endothelial cells express VE-cadherin at their lateral cell junctions (Navarro et al., 1998). VE-cadherin belongs to the type II cadherin subgroup (Lampugnani et al., 1997). Compared with classical cadherins, VE-cadherin amino acid sequence shares only 23% identity (Breier et al., 1996). In static cultures, VE-cadherin is distributed continuously around the cell junction at the midplane of the cell (Noria et al., 1999). These adherens junctions are important for maintaining stability of the monolayer under the physiological load of blood flow (Schnittler et al., 1997). The integrity of the monolayer is attributed in part to the capacity of VE-cadherin to mediate growth contact inhibition (Breviario et al., 1995; Navarro et al., 1995; Caveda et al., 1996). VE-cadherin complexes can also be very dynamic during processes such as vasculogenesis, angiogenesis and vascular permeability control (Navarro et al., 1995; Breviario et al., 1995; Vittet et al., 1996). Pro-inflammatory cytokines such as TNF-α and IFNγ and growth factors such as VEGF disrupt cell-cell adhesion in the endothelium by altering the association with actin filaments (Wong et al., 1999; Esser et al., 1998).

1.2.3 Regulation of Adherens Junctions

Stability of adherens junction complexes is regulated by phosphorylation of cadherins and catenins. A basal level of phosphorylation is essential for cell adhesion
while extensive phosphorylation of components of the adherens junction is correlated with a loss of stability, deterioration of adhesion sites and cell detachment allowing cell migration and cell motility (Soler et al., 1998). Tyrosine, serine and threonine phosphorylation target the catenins, specifically the armadillo catenins, and reduces their adhesion to the cytoskeleton which destabilizes the adherens junctions (Serres et al., 1997; Hamaguchi et al., 1993; Behrens et al., 1993). In v-src transfectants, where phosphorylation is excessive, adherens junctions are weaker and in many cases disrupted (Hamaguchi et al., 1993; Behrens et al., 1993). VE-cadherin is heavily tyrosine phosphorylated and mostly linked to p120 and β-catenin during early stages of junction assembly or when cells are released from tight confluency and migrate. When the junctions stabilize, tyrosine residues in VE-cadherin are dephosphorylated and p120 and β-catenin tend to detach from the complex and are replaced by plakoglobin (Lampugnani et al., 1997).

Modulation of the proteins associated with VE-cadherin complexes also occurs under the stimulus of shear stress. Plakoglobin and β-catenin both associate with VE-cadherin complexes when endothelial cells are quiescent and cobblestone. When cells are exposed to flow and gradually change shape, plakoglobin is lost from the junctions returning at later time points when the cells have completed their alignment with flow (Noria et al., 1999). It is suggested that the localization of β-catenin and plakoglobin may be related to the strength and/or integrity of the endothelial cell junctions (Schnittler et al., 1997).

Regulation of cadherin adhesiveness through phosphorylation and dephosphorylation events may be mediated by a balance between phosphatase and kinase activity. Phosphatases such as PTP1b, hPTPκ as well as kinases; src, lyn and yes and receptor kinases; EGF and c-erb-b2 localize to adherens junctions (Basalmo et al., 1996; Fuchs et al., 1996; Tsukita et al., 1991; Hoschuetzky et al., 1994; Kanai et al., 1995). PTP1-B-like tyrosine phosphatase associates directly with the cytoplasmic region of N-cadherin in neural retina cells. PTP-1B modulates cadherin function by regulating tyrosine phosphorylation of β-catenin (Basalmo et al., 1996). Displacement of PTP-1B from the cytoplasmic domain of N-cadherin by dephosphorylation results in enhanced tyrosine phosphorylation of β-catenin and loss of its association with cadherin and the
actin cytoskeleton (Basalmo et al., 1996; Basalmo et al., 1998). Similarly, in confluent, quiescent endothelial cells the cytosolic protein tyrosine phosphatase SHP2 binds selectively to β-catenin within VE-cadherin complexes. With the exposure to thrombin, SHP2 becomes phosphorylated and dissociates from the complex resulting in tyrosine phosphorylation of VE-cadherin-associated β-catenin, γ-catenin and p120 catenin (Ukropec et al., 2000).

Another phosphatase, receptor PTPµ, forms a complex with N-cadherin in retinal tissue. PTPµ regulates the reversible tyrosine phosphorylation of N-cadherin involved in the regulation of signals required for neurites to extend on N-cadherin. Overexpression of a catalytically inactive form of PTPµ inhibits N-cadherin mediated neurite outgrowth (Burden-Gulley et al., 1999). Receptor PTPµ also binds to and dephosphorylates the p120 catenin. The direct consequence of its dephosphorylation by rPTPµ remains to be determined although it is thought to be important in the regulation of the actin cytoskeleton (Zondag et al., 2000).

Growth factor receptors also associate with the cadherin/catenin complex and regulate junctional stability. The EGF receptor in epithelial cells binds directly to β-catenin. Activation of this receptor by EGF leads to phosphorylation of components of the adherens junctions that results in cell scattering activity (Hoschuetzky et al., 1994).

In addition to phosphorylation, junctions may also be regulated by the Rho family of GTPases. These signaling molecules which include Rho, Rac and Cdc 42 participate in the signal transduction mechanisms regulating the activity of the actin cytoskeleton. GTP bound forms of these proteins present at the cell membrane exert their functions by interacting with various effector molecules (Kaibuchi et al., 1999). One of the molecules that GTP bound Rac forms a complex with is IQGAP1. When Rac binds to IQGAP1 it prohibits IQGAP1 from binding to β-catenin and disrupting the catenin link to the cytoskeleton. As a result, α-catenin can bind unhindered to β-catenin and maintain junctional stability (Gumbiner, 2000). Epithelial cells overexpressing constitutively active Rac accumulated adherens junction proteins associated with actin fibers at cell-cell contact while a dominant negative Rac resulted in decreased localization of cadherin and catenin proteins at adherens junctions (Braga et al., 1997; Takaishi et al., 1997).
Similarly, when Rho was inactivated with inhibitors in endothelial cells there was a partial loss of cell-cell adhesion and VE-cadherin no longer associated with cell-cell junctions (Hordijk et al., 1999).

Cadherin function can also be regulated by proteolytic degradation. Matrix metalloproteinase activity at the cell surface can cleave the extracellular domain of N-cadherin to release a soluble 97kd fragment. The cleaved extracellular domain may activate a signal transduction pathway or alternatively, the remaining extracellular portion on the cell may induce a conformation change in the structure of the adherens junctions leading to downstream signaling events within the cell (Makrigiannakis et al., 1999).

### 1.3 N-Cadherin

#### 1.3.1 Expression

N-cadherin is expressed in various regions within the developing embryo such as the neural tube, notochord and mesoderm (Hatta et al., 1987). During development its expression and post-translational modifications are under precise spatial and temporal control. If N-cadherin is mutated or knocked out there is abnormal segregation, differentiation and assembly of cells. These mutations cause varying degrees of abnormalities within the developing embryo due to the ability of other cell adhesions molecules to compensate for the loss of function of N-cadherin. Expression of a truncated N-cadherin lacking its extracellular domain acts as a dominant negative form by competing with intact cadherins. In the developing retina of frog embryos these truncated N-cadherin molecules interfere with the formation of dendrites and axons. If any axons or dendrites are formed, their length is substantially reduced and the axons often lack growth cones (Riehl et al., 1996). Homozygous mutation in mice results in embryonic lethality by day 10 of gestation due to abnormal heart morphogenesis, neurulation and somitogenesis. (Radice et al., 1997). In adult tissue, N-cadherin is expressed in a wide variety of cells including lens epithelial cells, fibroblasts, endothelial cells, cardiac myocytes and striated muscle (Volk and Geiger, 1984; Knudsen et al., 1995; Salomon et al., 1992; Wheelock and Knudsen, 1991; Cifuentes-Diaz et al., 1998). Cells within neural
tissues also strongly express N-cadherin: oligodendrocytes, astrocytes and neurites (Payne et al., 1996; Wilby et al., 1999; Bixby and Zhang, 1990) and in these tissues N-cadherin is involved in mediating neuron-neuron adhesion, neuron-glia adhesion, and neurite growth and fasciculation (Bixby and Zhang, 1990, Redies, 1995).

1.3.2 Functions

N-cadherin has been implicated in a variety of functions ranging from morphogenetic movements to contact inhibition of cell migration.

1.3.2.1 Cell Survival

Cell aggregation mediated through cadherin homophilic and homotypic adhesion is linked to the prevention of apoptosis and cell survival. Rat granulosa cells (RGC) normally express N-cadherin; however, when these cells were cultured in the presence of N-cadherin disrupting antibody, there was a decrease in RGC aggregation and an increased rate of apoptosis. Apoptosis also occurred in these cells after administration of cAMP which caused a downregulation of N-cadherin through enzymatic cleavage of the extracellular domain (Makrigiannakis et al., 1999).

1.3.2.2 Contact Inhibition of Growth

N-cadherin mediated adhesion has been implicated in decreased proliferation mediated through contact inhibition. Ectopic expression of N-cadherin in a fibroblastic cell line (3Y1) induced tight association of the cells and growth arrest at G0/G1. (Wang et al., 1998). G1 phase cell cycle arrest was also demonstrated in CHO cells transfected with N-cadherin. Inhibitory antibodies directed against the extracellular domain of N-cadherin eradicated this effect, suggesting that adhesion mediated signals triggered by the clustering and/or immobilization of N-cadherin suppress cell growth (Levenberg et al., 1999).

1.3.2.3 Heterologous Cell Contact

Endothelial cells express N-cadherin, however, unlike VE-cadherin, N-cadherin is not clustered at endothelial cell-cell junctions (Salomon et al., 1992) and may be
excluded from junctions by a dominant activity of VE-cadherin (Navarro et al., 1998). Although N-cadherin does not mediate cell-cell contact it still retains its adhesive activity as it is concentrated at intercellular contacts in transfected Chinese Hamster Ovary cells (Salomon et al., 1992).

In vivo, rat vessels express N-cadherin within the endothelium as well as in a population of smooth muscle cell located immediately abluminal to the internal elastic lamina. Adhesion between co-cultured endothelial and smooth muscle cells is reduced when the cells are incubated with an antibody against N-cadherin; therefore N-cadherin may mediate interactions among heterologous cell types (Gilbertson-Beadling and Fisher, 1993).

1.3.2.4 Contact Inhibition of Migration

Contact inhibition of cell migration is mediated by N-cadherin in normal skeletal muscle myoblast, however this molecule does not suppress motility in subconfluent cells (Huttenlocher et al., 1998). An inverse correlation between the expression of N-cadherin and migration is also seen in neural crest cells in vivo. During embryogenesis, as neural crest cells begin to migrate, they detach from surrounding cells in the neural tube and have reduced levels of N-cadherin. As neural crest cells cease their migration and begin to re-aggregate to form the dorsal root ganglia, N-cadherin is re-expressed (Akitaya and Bronner-Fraser, 1992). Similarly, in vitro, neural crest cells do not establish extensive and stable intercellular contacts. Neural crest cells synthesize and express intact N-cadherin molecules on their surface, however most of these molecules are not connected with the cytoskeleton (Monier-Gavelle and Duband, 1995).

These disconnected N-cadherin complexes may be excluded from adherens junctions in the plasma membrane by a substratum signaling pathway. Cultured neural crest cells incubated with inhibitors of substratum adhesion were able to target newly synthesized N-cadherin complexes to cell-cell contacts. The pre-existing pool of N-cadherin molecules that was diffuse over the cell surface was not recruited to the adherens junctions after incubation with inhibitors of substratum adhesion, suggesting that it may have been irreversibly inactivated (Monier-Gavelle and Duband, 1997).
Integrins involved in migration may initiate signaling pathways that act as negative regulators of the expression and function of N-cadherin molecules. This action may keep cell-cell contacts transient by preventing accumulation of cadherin/catenin complexes into adherens junctions (Monier-Gavelle and Duband, 1997). Alternatively, integrins associated with stable adhesions may positively regulate cadherin expression. In myoblasts, enhanced integrin-mediated signaling through the ectopic expression of α5 or β1 integrin subunits upregulated N-cadherin expression and resulted in tight aggregates of cells. Similar aggregates were observed when paxillin was ectopically expressed. When these cells were incubated with low calcium or with an anti-N-cadherin antibody no aggregates were observed. This contact mediated inhibition of migration demonstrates a possible cross talk between integrin and cadherin receptor expression as ectopic expression of N-cadherin alone in these cells did not arrest motility (Huttenlocher et al., 1998).

1.3.2.5 Promotion of Migration

N-cadherin is present in non-motile cells such as myoblasts where it mediates strong, stable homotypic adhesions within the intercalated discs of the myocardium (Hertig et al., 1996) and in lens and mesothelium tissues whose junctions resemble those found in epithelial cells mediated by E-cadherin (Islam et al., 1996). N-cadherin is also expressed in cells that are more motile. Fibroblast cells do not display close cell-cell contacts yet express N-cadherin (Knudsen et al., 1995). N-cadherin is also expressed in a variety of cell types that are migrating. During myocardial embryogenesis N-cadherin expression is significantly increased in non-epithelial myocytes undergoing epithelial-mesenchymal transformation and migrating toward the endocardium. This increase is coincident with adherens junction formation that allows for cluster migration and clonal growth, both vital for the complete formation of the cardiac trabeculae and intercalated discs (Ong et al., 1998).

Similarly, in the developing chick embryo there is a switch from E to N cadherin when epiblast cells ingress through the primitive streak to form the mesoderm (Hatta and Takeichi, 1986; Edelman et al., 1983). This switch in cadherin expression from E to N also occurs in certain squamous epithelial cell lines that have become metastatic (Li et al.,
In normal epithelial cells, E-cadherin is expressed and promotes tight cell-cell adhesion that restricts cell movement. Originally it was believed that the loss of E-cadherin was responsible for the increased motility and invasion of these cancerous cells. However, certain N-cadherin expressing breast cancer cell lines that continue to express their endogenous cadherins (E & P) do not have an epithelioid morphology and are metastatic (Nieman et al., 1999). This finding suggests that N-cadherin overrides the ability of E-cadherin to maintain an epithelial phenotype in some cells. Cells transfected with N-cadherin retained their strong adhesive properties in short term aggregation assays and in clustered metastases in vivo, suggesting that reduction in cell adhesion is not the mode of action for N-cadherin induced invasiveness. Rather, it is likely that N-cadherin promotes a state of dynamic adhesion that allows both attachment and detachment of individual cells from the primary tumor and selective association with critical tissues such as the stroma or endothelium (Hazan et al., 2000). N-cadherin is also involved in transendothelial migration of melanoma cells. Specifically, N-cadherin is enriched at the contacts between endothelial cells and migrating melanoma cells in the vascular wall where normally VE-cadherin is found (Sandig et al., 1997). The rate of melanoma cell transmigration was severely decreased when antibodies against N-cadherin were used.

The involvement of N-cadherin in dynamic processes is also demonstrated in its adhesive role in the outgrowth of neurites (Bixby and Zhang, 1990). In addition to two other major cell adhesion molecules (CAMs), N-CAM and L1, N-cadherin is very important in promoting cell contact dependent axonal growth over a variety of cells such as astrocytes, oligodendrocytes and Schwann cells and their participation is spatially and temporally regulated (Doherty and Walsh, 1996). Axons from neurons extend and innervate their target by the homophilic binding of CAMs on the neuronal growth cone to CAMs on the surface of other axons or non-neuronal cells (Saffell et al., 1997). Antibodies against N-cadherin inhibit neurite outgrowth (Bixby et al., 1987; Tomaselli et al., 1988). CAMs may promote neurite outgrowth by stimulating calcium influx into neurons as neurite outgrowth by CAMs was inhibited completely by calcium channel antagonists and other agents that block or negate the effects of calcium influx into neurons (Doherty et al., 1991).
1.3.2.6 Signaling

CAMs may regulate the influx of intracellular calcium via the fibroblast growth factor receptor (FGFr). FGFr contains a highly conserved 20 amino acid sequence in the extracellular domain that shares homologies with sequences found in N-CAM, N-cadherin and L1 (Williams et al., 1994). The extracellular domain of FGFr contains a short sequence that shares homology with the “second” HAV region found only in the extracellular domain 4 of N-cadherin enabling a cis interaction (Doherty and Walsh, 1996). When the HAV sequence on FGFr is blocked with peptides or antibodies, neurite outgrowth on an N-cadherin substrate is inhibited (Williams et al., 1994).

The interaction of N-cadherin with FGFr activates the receptor and allows for the transfer of intracellular signals even without ligand. Activation is due to the ability of N-cadherin to dimerize FGF receptors. N-cadherin adherens junctions exist as dimers in the membrane that allow for the association of 2 FGFr with one cadherin dimer (Doherty and Walsh, 1996). Antibodies that prevent FGF from binding to its receptor do not inhibit neurite outgrowth stimulated by CAMs (Doherty et al., 1991); however, if FGFr lacks a tyrosine kinase domain (rendering it inactive), primary neurons are unable to extend neurite processes in response to stimulation by NCAM, N-cadherin, and L1 (Saffell et al., 1997).

FGFr activation through N-cadherin may also play a role in the ability of cancer cells of epithelial origin to infiltrate host tissue. Enhanced migration and production of MMP-9 was noted in a cancer cell line when cells were treated with FGF-2. Interestingly, only those cells that expressed N-cadherin displayed these results. This finding suggests that cell adhesion molecules, growth factor mediated signals and proteolysis of the extracellular matrix are all involved in the concerted action of cell migration, invasion and metastasis (Hazan et al., 2000).

1.4 Vascular Smooth Muscle Cells

1.4.1 Phenotypes of Smooth Muscle Cells

Smooth muscle cells are normally found in the media of blood vessels in a contractile and quiescent state. They contain minimal rough endoplasmic reticulum
(RER) and Golgi apparatus and a few free ribosomes located in the perinuclear region. These cells proliferate at a very low rate (less than 0.1% per day), a process that is controlled by complex interactions between growth stimulatory and inhibitory factors derived from endothelial cells and matrix constituents. The main function of quiescent vascular smooth muscle cells is to regulate the tone of the vessel wall through their contraction. As such, these cells are characterized by high levels of contractile proteins, as well as actin binding and structural proteins such as intermediate filaments; desmin and vimentin, high and low molecular caldesmon, light and heavy chains of smooth muscle myosin, smooth muscle-α-actin, calponin, vinculin and tropomyosin (Thyberg et al., 1990; Gabbiani et al., 1981).

Smooth muscle cells can also exhibit a synthetic phenotype as seen in development, repair and in pathological conditions such as restenosis and atherosclerosis. In these states, the smooth muscle cells respond to a multitude of growth factors and mitogens released from many different cell types including platelets, leukocytes, injured and dead smooth muscle cells and endothelial cells. These cells have a reduced capacity to contract and an increased capacity to divide, synthesize matrix and migrate. They are characterized by loss of myofilaments and formation of an extensive RER and a large Golgi complex. These organelles which take part in the synthesis and secretion of extracellular matrix components such as collagen type I, elastin and proteoglycans (Thyberg et al., 1990).

Synthetic smooth muscle cells express altered levels of differentiation marker proteins both at the protein and mRNA levels (Regan et al., 2000). Decreased proteins include smooth muscle myosin heavy chain, smooth muscle 22-α caldesmon, vinculin and desmin (Owens, 1995; Thyberg et al., 1990). Vimentin increases in intimal smooth muscle cells (Kocher et al., 1991). One of the most easily quantifiable differences between the two different phenotypes is the change in actin isoform levels. Four actin isoforms are expressed in fully differentiated smooth muscle cells: smooth muscle-α-actin, non-muscle-β-actin, non-muscle-γ-actin and smooth muscle-γ-actin. These isoforms are all products of separate genes that share a high degree of homology differing through their amino terminal peptides. Smooth muscle α-actin makes up 40% of total cell protein and over 70% of total actin in mature differentiated vascular smooth muscle
cells. Vascular smooth muscle cells undergoing a change in phenotype to a synthetic state show decreased expression of the α-isoform of smooth muscle actin and increased expression of the β and some γ isoforms (Thyberg et al., 1990; Gabbiani et al., 1984). The decreases in α-actin mRNA are present in the media of rat carotids as early as 8 hours after balloon injury, which suggests that cells begin to de-differentiate at early times after stimulus (Clowes et al., 1988). mRNA levels of *gax* (growth-arrest-specific-homeobox) are also decreased at early times after balloon injury. *Gax* is a homeobox transcription factor that is expressed at its highest levels in uninjured cardiovascular tissues. The decrease in *gax* at early time points after injury, when smooth muscle cells are de-differentiating suggests that is involved in maintaining gene expression of proteins associated with the contractile and quiescent state of smooth muscle (Weir et al., 1995).

Overall, the factors that control the modulation of smooth muscle cell phenotype are not fully understood. It may be affected by a combination of stimuli such as changes in cell interactions due to direct mechanical injury, alterations of extracellular matrix or altered hormone and growth factor levels. Smooth muscle cells in the media are surrounded by a basement membrane composed of laminin, heparan sulfate proteoglycan and type IV collagen. This membrane is thought to maintain the cell in a contractile state by establishing the required spatial arrangement of the components in the vessel wall. When this membrane is damaged the plasma membrane of the cell is exposed to different extracellular matrix proteins within the media as well as other macromolecules derived from the blood or produced locally by other cells in the vessel wall and causes an alteration in the cell's phenotype. Fibronectin and type I collagen promotes conversion of cultured cells from a contractile to a synthetic phenotype whereas elastin and laminin inhibit this change (Yamamoto et al., 1993; Hedin et al., 1988; Hedin and Thyberg, 1987). The extensive changes that smooth muscle cells undergo in protein expression and function are not mutually exclusive. Both developing and mature vessels are composed of smooth muscle cells that express a multitude of contractile proteins yet are still able to carry out functions of the synthetic cell such as synthesis of extracellular matrix proteins although at reduced levels (Owens, 1995).
1.4.2. Cultured Smooth Muscle Cells

Smooth muscle cells grown in culture exist in a range of phenotypic states. In the contractile state, cells are spindle shaped (150-200 μm x 10-15μm) with phase dense agranular cytoplasm and an oval nucleus containing several small, pale nucleoli and, depending on the tissue they were cultured from, frequently undergo spontaneous contractions (Campbell et al., 1981). Synthetic cells have a hypertrophic appearance with an increased cell surface to volume ratio and are sometimes difficult to distinguish from fibroblasts. Typically these cells grow in a ‘hill and valley’ configuration representing a multilayered pattern of densely populated smooth muscle cells (hills) and adjacent areas that are nearly devoid of smcs (valleys) (Powell et al., 1996). The extent of phenotypic modulation and its reversibility depends on several factors such as density of plating, percentage of serum, substrate on which the cells are grown, presence of other cells and number of doublings to achieve a confluent monolayer (Campbell et al., 1981; Thyberg et al., 1990; Campbell and Campbell, 1987).

1.5 Neointimal Thickening

1.5.1 Atherosclerosis

Atherosclerosis consists of a complex lesion within the intima of the artery that is composed of a variety of different cell types and matrix constituents. One of the key contributors to the progressive intimal thickening is the smooth muscle cell.

Intimal accumulation also occurs following therapeutic interventions to treat atherosclerosis. Percutaneous transluminal coronary angioplasty is used to restore the normal lumen diameter to atherosclerotic vessels. During angioplasty, both medial and intimal tissues are injured and vessels respond to this mechanical injury with a series of thrombotic, inflammatory and cellular events. In 30-40% of cases, these processes result in severe restenosis that leads to complete occlusion of the vessel by 6 months after the procedure (Davies and Hagen, 1994; Pauletto et al., 1994; Ferns et al., 1991). Restenosis is characterized by elastic recoil of the vessel, remodeling and neointimal thickening due to smooth muscle migration and proliferation.
1.5.2 Rat Carotid Model of Neointimal Thickening

Balloon catheter injury of the rat common carotid vessel is a well characterized model of neointimal thickening. In the uninjured vessel, a single monolayer of endothelial cells is present in the intima. The media of the common carotid vessel consists of 3 to 5 concentric layers of smooth muscle cells separated by evenly spaced elastic laminae. Smooth muscle cells in the media are encircled by a basement membrane composed of collagen type IV, laminin, entactin and heparan sulfate proteoglycans. The surrounding extracellular matrix is made up primarily of elastic laminae, fibrils of collagen type I and III, fibronectin and chondroitin/dermatan sulfate proteoglycans (Thyberg et al., 1990). The adventitia is composed of some fibroblasts, nerve fibers and microvessels enveloped by a dense collagenase matrix.

Balloon catheter injury strips the endothelial layer exposing the underlying internal elastic lamina that becomes covered by a layer of platelets and leukocytes. Because the common carotid has no branches endothelial regrowth will only occur from the proximal and distal ends of the vessel. Balloon injury also induces stretching and lysis of some of the vascular smooth muscle cells in the underlying layers. The mechanical injury in combination with factors produced and released at the site of injury activates the smooth muscle cells and leads to a change in their phenotype promoting migration and proliferation. The smooth muscle cells that migrate into the neointima are randomly arranged and are initially closely opposed to one another. The most luminal cells are flat, oriented parallel to the direction of blood flow and resemble the endothelium. Some cells form tight junctions, adherens junctions and gap junctions however, the majority of cells form intercellular clefts with wide gaps between them (Clowes et al., 1986; Kocher et al., 1991). These luminal cells continue to display a prominent secretory apparatus at later time points after injury while the bulk of the underlying cells in the neointima acquire a more quiescent phenotype with the return of contractile proteins (Thyberg et al., 1995).

The proliferation rate of smooth muscle cells also undergoes significant temporal regulation after balloon injury. In the normal vessel wall the rate of proliferation of medial smooth muscle cells as measured by thymidine labeling index is extremely low (0.06%). Within 2 days after arterial injury proliferation in the media increases towards a
maximum with a labeling index of 40-70%. (Gordon and Schwartz, 1987). Beginning approximately 4 days after injury medial smooth muscle cells migrate into the intima, and 50% of the medial smooth muscle cells that migrate undergo 3 rounds of division. These cells contribute 8/9 of the final intimal cell population whereas the other 50% migrate without further proliferation and make up 1/9 of the intimal cell population (Clowes and Schwartz, 1985). Proliferation of these intimal smooth muscle cells reaches a maximum at 7 days with total smooth muscle cell number reaching a maximum at 2 weeks. By 4 weeks proliferative activity throughout the arterial wall returns to baseline levels except at the luminal surface of the vessel. This continued proliferation does not contribute to an increase in overall cell number as proliferation equals cell loss (Clowes et al., 1986).

Studies following balloon denuded vessel for up to 12 months demonstrated that smooth muscle cell number remained relatively constant. These observations suggest injured vessel achieved steady state (Clowes et al., 1986). In addition to migration and proliferation, extracellular matrix deposition by smooth muscle cells is a key aspect of the development of the neointima, contributing to approximately 90% of the volume of the lesion (Strauss et al., 1996).

In conjunction with smooth muscle cell proliferation, endothelial regrowth from the proximal and distal ends of the vessel occurs in the weeks following angioplasty. Endothelial regrowth involves proliferation and migration and can be initiated by loss of contact inhibition, stretch, or growth factors secreted by endothelial cells, vascular smooth muscle cells and circulating cells. The extent of injury influences the regrowth of endothelial cells. Endothelial regrowth stops after several weeks (depends on species – 6-10 weeks in rat) when balloon catheter denudation involving medial cell death is performed, leaving the central third of the vessel devoid of endothelium even after 12 months (Clowes et al., 1986). This inhibition of endothelial regrowth is not due to contact inhibition by luminal smooth muscle cells or senescence of the endothelial cell but may be due to a plasma or platelet derived inhibitory factor (Reidy, 1988). If the endothelium is denuded without damaging the media, the endothelium maintains a sustained replication rate and complete endothelial regrowth is achieved (Lindner et al., 1989). In the areas devoid of endothelial cells, luminal smooth muscle proliferation continues at a level 50 fold greater than in control arteries and injured arterial segments covered by
endothelium. This may be supported by growth factors carried in the blood and that only the cells in the surface layer at able to respond (Clowes et al., 1983). Intimal smooth muscle cell proliferation still occurs even in areas covered by endothelium suggesting that endothelial cells may not be purely inhibitory (Van Belle et al., 1998).

### 1.5.3 Factors Involved in Neointimal Growth

Many different growth factors, hormones, prostaglandins, polypeptide mitogens, leukotrienes and cytokines are produced and released from various cell types in response to arterial wall stretch, cell injury and inflammation caused by balloon catheter injury. These factors may be derived from the blood, degranulated platelets, leukocytes, endothelial cells or smooth muscle cells. Some of these molecules exert only transient effects while others are chronic. Some molecules activate the same signaling pathways compounding the effects while others may mediate distinct processes. Some of the major factors involved are the following: PDGF, heparin, TGF-β1, FGF, Angiotensin II, TNF-α and IGF-1.

#### 1.5.3.1 PDGF

Following balloon denudation injury, subendothelial collagen is exposed causing platelets to adhere and to release Platelet Derived Growth Factor (PDGF) as well as other growth factors. Smooth muscle cells express PDGF receptors on their surface and binding of PDGF to these receptors can elicit a mitogenic and/or chemoattractive signal for smooth muscle cells (Clowes and Schwartz, 1985). Neutralizing antibodies against PDGF administered to rats after balloon catheter injury resulted in reduced migration of smooth muscle cells into the intima at early time points after injury (Jawien et al., 1992; Jackson et al., 1993; Ferns et al., 1991)

#### 1.5.3.2 Heparin

Heparin is secreted primarily by post confluent endothelial cells but also by smooth muscle cells (Liu et al., 1989). One of its major roles is to maintain smooth muscle cells in a quiescent growth state by inhibiting their proliferation and migration (Majack and Clowes, 1984). It is postulated that its mode of inhibition of migration is
through suppression of the expression of matrix degrading enzymes (Clowes et al., 1992). After balloon injury heparin levels are decreased in the vessel by two methods: the endothelium is removed (Castellot and Karnovsky, 1987) and heparinase is released from the lysosomes of platelets which cleaves and inactivates local heparin (Campbell and Campbell, 1987). With decreased heparin levels the balance between inhibitory and stimulatory signals is disrupted leading to proliferation and migration. Balloon injured arteries infused with heparin had a marked reduction in neointimal proliferation (Karnovsky and Clowes, 1977). Injection of heparin immediately after injury depletes the arterial wall of bFGF and this mechanism may be responsible for the inhibition of medial smooth muscle cell proliferation (Lindner et al., 1992).

1.5.3.3 TGF-β1

Transforming growth factor-β1 (TGF-β1) stimulates the synthesis of extracellular matrix components, and also regulates cell replication (Rasmussen et al., 1995). TGF-β1 is secreted by smooth muscle cells, endothelium and platelets in a latent form and requires plasmin for activation. TGF-β1 has been identified in animal models of restenosis and in human intimal hyperplastic lesions. mRNA for TGF-β1 increases within 6 hours after balloon injury in rat carotid arteries and remains markedly increased up to 7 days (Majesky et al., 1991). The presence of a neutralizing antibody against TGF-β1 reduced proteoglycan synthesis by the cells from injured arteries in culture (Rasmussen et al., 1995).

1.5.3.4 bFGF

Increased levels of basic fibroblast growth factor (bFGF) are present in the arterial wall after vascular injury due to the release of stored bFGF from damaged cells (Lindner and Reidy, 1993). bFGF is involved in the early replication of medial smooth muscle cells after balloon catheter injury (Lindner et al., 1991). Administration of an antibody to bFGF blocks the smooth muscle cell proliferation that occurs 48 hours after injury (Lindner and Reidy, 1991). This growth factor is also responsible for replication of the endothelium. Balloon injured vessels express high levels of bFGF localized to the
endothelium within the first few weeks and becomes almost absent at later time points when replication of the endothelium has stopped (Lindner et al., 1989).

1.5.3.5 Angiotensin II

Angiotensin II functions as a potent vasoconstrictor and growth factor in vascular smooth muscle cells. Angiotensin II binds to its receptors on smooth muscle cells activating signal transduction pathways leading to cellular hypertrophy (Thyberg et al., 1990) with an increase in protein content but with no effects on cell proliferation (Geisterfer et al., 1988). Alternatively, another group reported that smooth muscle cells do proliferate in response to angiotensin II (Campbell-Boswell and Robertson, 1981). The disparities between responses may be due to exposure times of angiotensin II and/or cell types. Interestingly, in vivo balloon injury studies using an angiotensin-converting enzyme inhibitor (that prevented the formation of angiotensin II), showed a decrease in neointimal size due to a reduction in both matrix formation and smooth muscle cell number (Powell et al., 1989).

1.5.3.6 TNF-α

Tumor necrosis factor-α is a cytokine that is released by inflammatory cells at sites of vascular injury and is expressed in vascular smooth muscle cells after balloon injury (Goetze et al., 1999). TNF-α binding to its receptor leads to smooth muscle cell migration through a MAPK dependent pathway. TNF-α induced rapid depolymerization of F-actin stress fibers and the disappearance of vinculin from focal adhesion in vascular smooth muscle cells (Jovinge et al., 1997).

1.5.3.7 IGF-1

IGF-1 is a mitogen and a potent chemoattractant for vascular smooth muscle cells (Wang et al., 1997). Mitogenic and chemotactic actions of IGF-1 are mediated through the IGF-1 receptor, a transmembrane tyrosine kinase that is abundantly expressed in vascular smooth muscle cells (Duan et al., 1999). The intracellular pathways mediating these responses are through PI3kinase as treatment of vascular smooth muscle cells with
wortmannin abolished IGF-1 induced proliferation and substantially reduced migration (Duan et al., 1999).

1.5.4 Migration of Smooth Muscle Cells

The migration of vascular smooth muscle cells from the media to the intima is a complex process that involves the coordination of several events including the proteolysis of extracellular matrix components, cell-matrix and cell-cell adhesion and de-adhesion events in order to overcome the surrounding barriers of matrix and cells.

1.5.4.1 Integrins

One of the major molecules involved in migration is the integrins. They are transmembrane heterodimeric receptors that link the cell to the extracellular matrix. A single receptor is comprised of an α and β subunit that are non-covalently associated with one another. Different combination of subunits bind to different extracellular matrix proteins that contain the RGD (arginine, glycine, asparagine) amino acid sequence. The binding of ligand results in integrin clustering leading to the formation of focal adhesion plaques in which the cytoplasmic portion of integrin subunits interact with the termination sites of actin stress fibers, and various actin associated and signaling proteins (Sanders et al., 1998). Integrins are involved in promoting cell adhesion and/or migration depending on the subunits expressed on the cell surface and the available extracellular ligands. Quiescent smooth muscle cells are surrounded by a basement membrane consisting of type IV collagen and laminin that is rich in heparan sulphate proteoglycans.

Integrins α1β1 and α2β1 bind to these extracellular matrix molecules and along with soluble mediators maintain the cell in a quiescent state (Newby and Zaltsman, 2000). After balloon injury, the media is damaged resulting in disruption of the basement membrane. Smooth muscle cells are then exposed to fibronectin and monomeric type I and III collagen as well as newly synthesized extracellular matrix proteins. These ligands bind to integrin receptors α5β1 and αvβ3 and provide signals for migration and proliferation (Newby and Zaltsman, 2000). The αvβ6 integrin receptor has also been associated with a migratory function (Kumar, 1998). The differences in receptor function are mediated through differences in receptor affinity, avidity or expression (Preissner et
al., 1997). In cultured aortic smooth muscle cells when the extracellular domain of β1 is blocked the cells exhibit enhanced migratory behavior. Conversely, if the β3 integrin chain is blocked, migratory behavior is suppressed and the cells become more stationary in culture (Clyman et al., 1992). It is postulated that higher avidity β1 integrin receptors found centrally under the cell and peripherally at the spreading edge of the cell have a dominant role in anchoring the cell to the matrix. In contrast, αvβ3 receptors, present only around the periphery of the cell facilitate cell spreading and migration (Clyman et al., 1992).

In human atherosclerotic lesions and restenotic lesions as well as in animal models of intimal hyperplasia αvβ3 and its ligand, osteopontin, are upregulated at early time points after injury and decreased at later time points (Corjay et al., 1999). Neutralizing antibodies to osteopontin or RGD antagonists limit neointima formation. These results suggest that interactions between certain integrins and their ligands at early time points may mediate adhesion and migration of smooth muscle cells (Corjay et al., 1999).

Other provisional matrix proteins are also upregulated in the newly forming neointima. Fibrin levels are high in the neointima at early time points after injury. α5β1 and αvβ3 present on smooth muscle cells bind to fibrin and use it as a ligand in order to spread and migrate into fibrin gels (Ikari et al., 2000). Levels of type VIII collagen levels are also elevated in the neointima early after injury. Once smooth muscle cells have reached the intima the levels of type VIII collagen decrease and are replaced by a more stable, permanent matrix consisting of fibrillar type I and III collagen and elastin (Bendeck et al., 1996). A receptor for several of the collagens is the Dicoidin Domain Receptor (DDR). It is expressed on neointimal smooth muscle cells after injury. By regulating the attachment to collagen and increasing the production of MMPs, DDRs are postulated to be critical for the migratory and proliferative activity of smooth muscle cells as DDR1 null mice had reduced neointimas after mechanical injury (Hou et al., 2001).

The mechanics behind cell migration have been extensively characterized in fibroblast cells. The fibroblast model of migration involves attachment at the leading edge of the cell through focal adhesions, cell contraction where the cell remains fixed as
the cell migrates over the focal adhesions and detachment at the rear end (Smilenov et al., 1999). The attachment and detachment at the leading and trailing edge of the cell is mediated by integrins undergoing a cycle of formation and dissolution. As cells detach, integrins are either released into the extracellular matrix or are internalized (Friedl and Brocker, 2000). Cell shape change and contraction involve active remodeling of the actin cytoskeleton through polymerization and depolymerization. These processes are very complex and are mediated by a variety of proteins and pathways within the cell (Goetze et al., 1999, Sobieszek, 1995). Growth factors such as PDGF have been implicated in the signaling pathways involved in cell motility and actin remodeling. PDGF causes a rapid and reversible disappearance of vinculin from focal adhesion plaques as well as a disruption of actin filaments bundles in cultured cells (Herman et al., 1987).

1.5.4.2 Matrix Degradation

In addition to cytoskeletal rearrangements and dynamic contacts with the extracellular matrix smooth muscle cells must also be able to degrade components of the extracellular matrix in order to migrate efficiently. After arterial injury, newly migrating smooth muscle cells express both tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) on their surface (Jackson et al., 1993). tPA and uPA convert plasma derived inactive plasminogen to active plasmin which can then degrade a variety of matrix proteins and activate metalloproteinases. The uPA receptor is also expressed on the migrating cell concentrated at the leading edge of the cell where movement occurs (Okada et al, 1995). The importance of these molecules in promoting migration is demonstrated in uPA deficient mice which displayed reduced neointimal formation (Carmeliet and Collen, 1998).

Another group of matrix degrading enzymes involved in smooth muscle cell migration after injury is the matrix metalloproteinases (MMPs). MMPs belong to a family of zinc-dependent extracellular matrix degrading enzymes comprised of cell surface (membrane-type) MT-MMPs and non-surface associated MMPs. MMPs are secreted from cells as latent enzymes, which can subsequently be converted to active enzymes by proteases or MT-MMPs (Newby and Zaltsman, 2000). Once they are activated, MMPs degrade the encaging extracellular matrix that surrounds smooth muscle
cells allowing localized cell migration into the neointima. Natural inhibitors of MMPs; the tissue inhibitor of matrix metalloproteinases (TIMPs) are also present within the vessel wall and function to balance the proteolytic degradation exerted by MMPs (Dollery et al., 1995). MMP activity is normally low or undetectable in uninjured vessels however gelatinolytic activity increases after injury contributing to the reshaping of the extracellular matrix and vessel remodeling. Active MMP-9 levels increase within 24 hours after ligation of rat carotids and remain elevated coincident with the time course of smooth muscle cell migration (Bendeck et al., 1994). MMP-2, which is constitutively expressed in the vessel wall, also increases after ligation although at a later time point suggesting its involvement with carotid artery shrinkage (Godin et al., 2000). The importance of MMP activity in smooth muscle cell migration is demonstrated with treatment of balloon injured vessels with a metalloproteinase inhibitor. These vessels had nearly complete inhibition of smooth muscle cell migration resulting in reduced neointimal thickening at early time points after injury (Bendeck et al., 1996).
1.6 Objectives

Migration of smooth muscle cells is a complex process. Cell adhesion molecules, chemotactic factors, cytoskeletal proteins and matrix degrading enzymes are only a few of the proteins that are tightly regulated in order to coordinate the movement of these cells into the neointima. Events occurring pre- and post-migration are also extensively regulated. Phenotypic modulation of smooth muscle cells occurs prior to migration with widespread changes in the proteins and structures expressed within the cell which allows it to adopt different functions. Migration and proliferation of these de-differentiated cells leads to the development of neointimal thickening. As these processes are occurring, the growing neointima undergoes extensive remodeling. Extracellular matrix constituents are formed and deposited by the smooth muscle cells, basement membranes are re-formed around cells, and cell-matrix and cell-cell interactions are made and broken. Gradually these processes wane, and the neointima becomes more stable with the bulk of the smooth muscle cells reverting back to a more contractile phenotype. Many unanswered questions surround the processes of phenotypic modulation and migration of smooth muscle cells. Are cell-cell adhesions important in maintaining phenotypic states? How are they regulated as the cell undergoes phenotypic alterations and migrates? Are they extensive in the stable neointima and how are they regulated? The overall goal of this study is to characterize N-cadherin adherens junction complexes in vascular smooth muscle cells and determine how this junction is modulated as smooth muscle cells undergo phenotypic alterations and migrate during repair processes. Examining these processes will lead to a greater understanding of the mechanics of smooth muscle cell migration and the dynamic interaction between the cell and its environment after various migratory and proliferative cues.
CHAPTER 2

N-Cadherin Adherens Junctions in Vascular Smooth Muscle Cells: Role in Migration
2.1 Introduction

Smooth muscle cells express a wide variety of integrin receptors that mediate basal cell adhesion contacts. Certain receptors have been associated with the quiescent state of the cell promoting stable cell-matrix interactions while others have been implicated with migration (Newby and Zaltsman, 2000; Kumar, 1998). By contrast, cell-cell adhesions have not been extensively studied in smooth muscle cells and how they are regulated during migration. N-cadherin has been found in a wide range of cells including neurites, oligodendrocytes, astrocytes, endothelial cells, muscle (striated and cardiac), lens epithelial cells and fibroblasts (Bixby and Zhang, 1990; Payne et al., 1996; Wilby et al., 1999; Salomon et al., 1992; Cifuentes-Diaz et al., 1995; Wheelock and Knudsen, 1991; Volk and Geiger, 1984; Knudsen et al., 1995). Its role in these various types is varied. In cells that do not display close cell-cell contacts, such as fibroblasts, N-cadherin is capable of mediating labile and stable interactions (Knudsen et al., 1995). N-cadherin is also preferentially expressed in cells that are actively migrating (Nieman et al., 1999; Ong et al., 1998; Islam et al., 1996; Hazan et al., 2000). I hypothesized that N-cadherin is localized to focal adherens plaques mediating adhesions between contacting cells and promotes the migration of smooth muscle cells.

2.2 Materials and Methods:

2.2.1 Immunostaining of Cultured Cells

Porcine aortic smooth muscle cells between passages 4 and 9 were plated on glass cover slips (22x 22mm) in medium 199 with 5% FBS, 1% fungazone and 1% penicillin streptomycin and allowed to reach confluence in a 37°C incubator with 5% CO2. Cells were rinsed with Phosphate Buffered Saline (PBS) containing Ca2+ and Mg2+ 3x5 minutes then fixed with 3% paraformaldehyde for 20 minutes. Cells were rinsed with PBS 3x5 minutes, incubated with 0.2% Triton X-100 for 5 minutes, then rinsed with PBS 3x5 minutes. Cells were incubated with mouse monoclonal anti-N-cadherin antibody (Transduction Laboratories) in a dilution of 1:50, goat polyclonal anti-α-catenin antibody 1:50 (Santa cruz), goat polyclonal anti-β-catenin antibody 1:50 (Santa Cruz), goat
polyclonal anti-plakoglobin antibody 1:50 (Santa Cruz) or anti-smooth muscle-α-actin antibody 1:200 (Sigma) for 1 hour. Cells were rinsed with PBS 3x5 minutes then incubated with an FITC conjugated donkey anti-mouse or anti-goat antibody (Jackson ImmunoResearch Laboratories) in a dilution of 1:50 for 30 minutes in the dark. Some cells were co-incubated with rhodamine phalloidin (Molecular Probes) in a dilution of 1:20 to stain the actin filaments and with FITC conjugated donkey-anti-mouse or anti-goat antibody (Jackson ImmunoResearch Laboratories) in a dilution of 1:50 for 30 minutes in the dark. The coverslips were then rinsed with PBS 3x5 minutes, mounted on glass microscope slides with glycerol:PBS (9:1) and viewed under a Biorad 1024 laser scanning confocal microscope (Nikon X60 oil immersion objective with 1.4 numerical aperture). FITC was excited at a wavelength of 488 nm, and a band pass filter (506-538 nm) was used to detect fluorescence. Rhodamine was excited at 568 nm, and fluorescence was detected between 589 and 621 nm.

2.2.2 Immunoblotting

Porcine aortic smooth muscle cells between passages 4-9 were grown to confluence then lysed in a buffer containing 0.5% SDS, 10% Nonidet P40 and 10% Na-deoxycholate. 10µg of total protein was separated on an 8% SDS-PAGE gel. Gels were transferred onto a PVDF membrane. Membranes were blocked for 1 hour in 5% non-fat milk then incubated with mouse monoclonal anti-N-cadherin antibody 1:2000 (Transduction Laboratories) or mouse monoclonal anti-pan cadherin antibody 1:500 (Sigma) diluted in 2.5% non-fat milk for 1 hour. Membranes were washed 3x15 minutes with Tris Buffered Saline + 0.1% Tween 20 (TBST), incubated with sheep anti-mouse HRP conjugated secondary antibody (Amersham) 1:1000 diluted in 2.5% non-fat milk for 1 hour, washed 3x15 minutes with TBST, incubated with ECL western blotting detection system (Amersham Pharmacia Biotech) for 1 minute and developed.

2.2.3 In Vitro Wound Migration Assay

Porcine smooth muscle cells between passages 4-9 were grown on 35mm dishes in medium 199 with 5% FBS, 1% fungazone and 1% penicillin streptomycin. When cells
reached confluency, either mouse IgG (Sigma) 10μg/ml or monoclonal anti-N-cadherin antibody, A-Cell-Adhesion-Molecule (A-CAM) (Sigma) 10μg/ml were added to the plates 1 day prior to wounding. The following day, a pipette tip was drawn across the cells creating a wound approximately 800μm wide. Wound edge growth and wound width were measured from phase contrast pictures at 0, 12, 24 and 48 hours after wounding. Mean values of wound edge growth and percent wound width were subjected to 1 way ANOVA with Dunnet tests (n=5).

2.3 Results

2.3.1 Distribution of Adherens Junction Proteins in Vascular Smooth Muscle Cells

Confirmation of the lack of fibroblast contamination in the primary cultures of porcine aortic smooth muscle cells was observed by the pattern of immunofluorescent staining with a monoclonal antibody against smooth muscle-α-actin. This protein is the most predominant in smooth muscle cells and was present in long continuous fibers throughout the length of the cell (Figure 2.1). N-cadherin was densely localized at the tips of actin projections between contacting cells (Figure 2.2). This protein was found midway between the apical and basal surface of the cell (Figure 2.3). Porcine aortic smooth muscle cells also expressed the three cytoplasmic catenins; α-catenin, β-catenin and plakoglobin, which were also localized at the cell-cell contacts (Figure 2.4).

2.3.2 Pan-Cadherin and N-Cadherin Immunoblots

Smooth muscle cell lysates were immunoblotted using a monoclonal pan-cadherin antibody targeting the cytoplasmic domain of the classical cadherin family. A single band was detected at 135kd (Figure 2.5a). This band was confirmed to be N-cadherin through the use of a monoclonal antibody against N-cadherin detecting a doublet at 135kd (Figure 2.5b).
2.3.3 Inhibition of Smooth Muscle cell Migration with an N-cadherin Antibody

Pre-incubation of porcine aortic smooth muscle cells with an N-cadherin antibody (Figure 2.6 row A) caused a significant reduction in the size and shape of smooth muscle processes extending into the wounded area at 12 hours, 24 hours and 48 hours after injury. Cell processes were shorter and more blunted. In contrast, cells pre-incubated with mouse IgG (Figure 2.6 row B) had long thin processes that extended into the wound. Statistical analysis of percent wound width against time showed that there were significant differences (p<0.05) between the two groups at 24 and 48 hours after wounding. Specifically, at 48 hours, the N-cadherin antibody group closed the wound by 30%, while the control group closed it by 50% (Figure 2.7). Wound edge growth versus time also showed significant differences at 24 and 48 hours after wounding between the two groups (30% and 40% respectively) (Figure 2.8).
Figure 2.1: Confocal micrograph of porcine aortic smooth muscle cells immunostained to detect smooth muscle-α-actin. The image is a projection of optical sections collected at a separation of 0.25μm from the apical to the basal surface of the cell. Smooth muscle-α-actin is present throughout the cell in long continuous fibers. Scale bar measurement is μm unless otherwise stated.
Fig 2.2: Confocal micrograph of porcine aortic smooth muscle cells immunostained to detect N-cadherin. The image is a projection of optical sections collected at a separation of 0.25 μm from the apical to basal surface of the cell. N-cadherin is localized to the tips of cell projections making contact with neighboring cells.
Figure 2.3: Confocal micrographs of optical sections collected at a separation of 0.25 μm from the basal (A) to apical (H) surface of porcine aortic smooth muscle cells. Cells are immunostained to detect N-cadherin. Adherens junction plaques are localized to the midplane of contacting cells (shown by arrow).
Figure 2.4: Confocal micrograph projections of porcine aortic smooth muscle cells immunostained to detect N-cadherin (A), α-catenin (B), β-catenin, (C) and plakoglobin (D) - all in green. Actin fibers are stained with rhodamine phalloidin (red). The images are projections of optical sections collected at a separation of 0.25 μm from the apical to basal surface of the cells. Arrows denote adherens junction complexes composed of the 4 proteins localized to the tips of actin filaments at cell-cell contacts.
Figure 2.5: Immunoblots of smooth muscle cell lysates probed with an antibody to pan-cadherin (A) and N-cadherin (B). A single band was detected at 135kd with pan-cadherin and a doublet was detected at 135kd with N-cadherin.
Figure 2.6: Phase contrast pictures at the wound edge of porcine aortic smooth muscle cells incubated with a function blocking N-cadherin antibody (row A) and mouse IgG (row B) at 0, 12, 24 and 48 hours after wounding. Cells incubated with the N-cadherin antibody have shorter, blunted cell processes extending into the wound area compared to controls.
Figure 2.7: Percent wound width measured against time of porcine aortic smooth muscle cells incubated with mouse IgG (yellow) or N-cadherin antibody (blue-crosshatch). At 24 and 48 hours wound width was significantly larger with cells incubated with the N-cadherin antibody compared to control.
Figure 2.8: Wound edge growth measured against time of porcine aortic smooth muscle cells incubated with mouse IgG (yellow) or N-cadherin antibody (blue-crosshatch). Cells incubated with the N-cadherin antibody had significantly reduced wound edge growth at 24 and 48 hours after wounding compared to control cells.
2.4 Discussion

Porcine smooth muscle cells in culture displayed the typical hill and valley morphology with extensive overgrowth of cells. Cell-cell adhesion between these cells was mediated by N-cadherin adherens junctions. Western analysis of smooth muscle cells probed with an antibody to pan-cadherin detected only one band at approximately 135kd suggesting that smooth muscle cells may express only one type of cadherin from the classical family. This band was confirmed to be N-cadherin by probing lysates with a monoclonal antibody to N-cadherin that detected a doublet at 135kd. The upper band of the doublet may be a glycosylated or phosphorylated precursor protein of N-cadherin that is proteolytically cleaved.

Immunofluorescent staining showed that the distribution of N-cadherin and its associated cytoplasmic linker proteins were localized at the tips of actin projections making contact with neighboring cells. Positive nuclear staining was noted particularly with the N-cadherin antibody. This may be due to antibody cross-reactivity with nuclear proteins. Confocal micrograph projections of optical sections collected at a separation of 0.25 μm from the basal to the apical surface of the cell demonstrated that the adherens junctions composed of N-cadherin were specifically localized at the midplane of the cell.

Cultured smooth muscle cells are normally found in a synthetic state which is a phenotype that is also present in vivo at early time points after balloon injury when the cell are actively migrating. The migration of these synthetic smooth muscle cells in culture was inhibited after administration of a blocking antibody that targeted both newly forming and existing N-cadherin junctions. In general, by 48 hours after wounding control cells closed the wound area by 50% while cells pre-incubated with N-cadherin antibody only closed it by 30%. At 10μg/ml the blocking antibody effectively diminished the ability of smooth muscle cells to migrate. At higher concentrations not only is the effect of the antibody more pronounced at the wound edge but also throughout the entire dish as cells became very spindly with few contacts between cells (data not shown).

In highly adhesive cell types, reduced cellular adhesion is often associated with increased migration (Calof and Lander, 1991; Huttenlocher et al., 1996), however there is
not a direct correlation between adhesivity and cellular ability to migrate. Loss of β1 integrin in embryonic stem cells results in impaired migration (Fassler and Meyer, 1995). My in vitro wounding experiment is one example where decreased cellular adhesion may impede the cell's ability to migrate. The mechanism mediating this reduced migration is at present unclear. Migration of cells requires dynamic formation and dissolution of cell adhesions. In addition, adhesive interactions must be able to generate the traction and force required for cell movement. N-cadherin may promote both labile and stable interactions with neighboring cells. These transient contacts may enable cells to use each other as anchors on their path to migration. This type of mechanical coupling between cells is common in fibroblasts in the form of viscoelastic tension. Transmission of this force between adjacent cells is reported to be mediated by N-cadherin adherens junctions (Ragsdale et al., 1997).

In addition to N-cadherin’s possible role in transmitting forces between adjacent cells through cell-cell contact promoting migration, it may also be transmitting intracellular signals involved in the regulation of migration. Studies have shown that integrin signaling promotes the downregulation of N-cadherin in neural crest cells that are actively migrating (Monier-Gavelle et al., 1997). Results from my experiment suggest that the integrin/cadherin cross-talk signaling pathway may be working in the opposite direction. When the extracellular domain of N-cadherin is blocked by antibodies it may prevent the clustering of cadherins on the cell surface, this lack of clustering may feedback to intracellular pathways with the end result of upregulating the level of integrins associated with stable adhesions preventing the cell from migrating.
CHAPTER 3

Neointimal Modulation of N-Cadherin Adherens Junctions in Vascular Smooth Muscle Cells After Balloon Catheter Injury
3.1 Introduction

The function of N-cadherin varies from cell type to cell type. Increased levels of N-cadherin have been associated with contact inhibition of cell growth that involves cell cycle arrest in Chinese hamster ovary cells (Levenberg et al., 1999), increased motility in breast carcinoma cells (Nieman et al., 1999) and increased migration of epithelial-mesenchymal myocytes in the developing endocardium (Ong et al., 1998). Results from chapter 2 implicate a possible role for N-cadherin in migration of cultured synthetic vascular smooth muscle cells. As a result, I hypothesized that N-cadherin is involved in the migration of medial smooth muscle cells into the neointima in response to balloon catheter injury of the rat carotid artery.

In vivo, smooth muscle cells alter their phenotype from contractile to synthetic after balloon catheter injury. In this state, smooth muscle cells migrate, proliferate and deposit extracellular matrix constituents into the neointima. These processes are transient as the neointima reaches its maximal thickness by approximately 8 weeks. At this time point, the bulk of the smooth muscle cells within the neointima have reverted to a more contractile phenotype and have stabilized their interactions with matrix and surrounding cells.

One type of junction that undergoes reorganization depending on the state of the cell is the adherens junction complex. In cultured adult rat cardiomyocytes expressing N-cadherin adherens junctions catenin expression and distribution change as the cells undergo modulation. Newly synthesized β-catenin complexes are more abundant during the first stages in culture after myocyte isolation, while newly synthesized plakoglobin containing complexes progressively accumulate during the morphological changes of the myocytes (Hertig et al., 1996). Endothelial cells also display differential expression of these two catenin proteins. β-catenin is localized to junctions that are undergoing dynamic changes while higher levels of plakoglobin are present at the junctions when the cells are in a confluent monolayer and are stable (Lampugnani et al., 1995). In the balloon injury model, junctional complexes within smooth muscle cells may reorganize as the cell undergoes phenotypic alteration and migrates. Even without complete reversion of phenotype, a stabilization of the neointima may involve junctional
reorganization. As intimal growth and subsequent stabilization takes place there could be changing levels of N-cadherin, plakoglobin and β-catenin within the adherens junction complex. I hypothesized that β-catenin is the dominant catenin in the adherens junction complex as the cells migrate while plakoglobin levels increase at later time points when junctions have stabilized.

### 3.2 Materials and Methods

#### 3.2.1 Balloon Catheter Injury

Male Sprague-Dawley rats 350-400g were anesthetized with an intraperitoneal 0.70 ml injection of xylazine (3.2mg/ml) & ketamine (49mg/ml). A midline incision was made in the neck and the left external carotid artery was dissected free of surrounding tissue. A 2F Fogarty balloon tipped catheter was introduced via the left external carotid artery, inflated and passed three times through the common carotid artery. Rats were killed using the euthanasia solution T61 (1ml/rat), at 1 week, 3 weeks and 8 weeks after injury.

#### 3.2.2 Immunoblotting

Control (uninjured) and injured carotid arteries were harvested, rinsed with saline and frozen at -80°C. Arteries were ground up and lysed in buffer containing 0.5% SDS, 10% Nonidet P40 and 10% Na-deoxycholate. 20μg of total protein was separated on a 10% SDS-PAGE gel. Gels were transferred onto a PVDF membrane, blocked in 5% non-fat milk for 1 hour, then incubated with mouse monoclonal anti-N-cadherin 1:2000 (Transduction Laboratories), mouse monoclonal anti-β-catenin 1:1000 (Santa Cruz), mouse monoclonal anti-plakoglobin 1:2000 or mouse monoclonal anti-smooth muscle-α-actin 1:20000 (Sigma), diluted in 2.5% non-fat milk for 1 hour. Membranes were washed 3x15 minutes with TBST, incubated with sheep anti-mouse HRP 1:1000 conjugated secondary antibody (Amersham) diluted in 2.5% non-fat milk for 1 hour, washed 3x15 minutes with Tris buffered saline + 0.1% Tween 20 (TBST), incubated with ECL western blotting detection system (Amersham Pharmacia Biotech) for 1 minute and developed.
3.2.3 Cross-Section Immunostaining

Control (uninjured) and injured carotid arteries were perfusion fixed with 3% paraformaldehyde at constant physiological pressure for 4 minutes then rinsed with PBS. The arteries were paraffin embedded and cut into 7 μm cross-sections. Slides were deparaffinized in xylene for 10 minutes then rehydrated in gradients of ethanol. Slides were incubated with 0.2% triton X-100 for 5 minutes then rinsed 3x5 minutes with PBS containing Mg2+ and Ca2+. Slides were incubated with 10% horse serum for 20 minutes then incubated with a primary antibody that was diluted in 5% horse serum for 1 hour. Primary antibodies used were: goat polyclonal anti-β-catenin 1:50 (Santa Cruz), mouse monoclonal anti-plakoglobin 1:50 (Transduction Laboratories), goat polyclonal anti-N-cadherin 1:50 (Santa Cruz) and mouse monoclonal anti-smooth muscle-α-actin 1:200 (Sigma). The slides were rinsed with PBS 3x5 minutes then incubated with a CY3 conjugated donkey anti-goat or anti-mouse antibody 1:100 (Jackson Immuno Research Laboratories) diluted in 5% horse serum for 30 minutes in the dark. The slides were then rinsed with PBS 3x5 minutes, coverslipped with glycerol:PBS 9:1 and viewed under a Biorad 1024 laser scanning confocal microscope (Nikon X60 oil immersion objective with 1.4 numerical aperture). Elastin was excited at a wavelength of 488 nm, and a band pass filter (506-538 nm) was used to detect fluorescence. CY3 was excited at 568 nm, and fluorescence was detected between 589 and 621 nm.

3.2.4 En face Immunostaining

Control (uninjured) and injured carotids were perfusion fixed with 3% paraformaldehyde at constant physiological pressure for 4 minutes then rinsed with PBS. The adventitia was removed enabling the artery to lie flat with the intimal layer facing up after being opened longitudinally. Arteries were incubated with 0.2% Triton X-100 for 5 minutes then rinsed 3 x 5 minutes with PBS containing Mg2+ and Ca2+ and incubated with the following primary antibodies for 1 hour: goat polyclonal anti-β-catenin antibody 1:50 (Santa Cruz), goat polyclonal anti-N-cadherin 1:50 (Santa cruz) or goat polyclonal anti-plakoglobin antibody 1:50 (Santa Cruz). Arteries were rinsed with PBS 3x5 minutes then incubated with a CY3 conjugated donkey anti-goat antibody (Jackson Immuno
Research Laboratories) in a dilution of 1:100 for 30 minutes in the dark. Following incubation, and 3x5 minute rinses with PBS, whole mount preparations with intima side up were cover slipped with glycerol:PBS 9:1 and viewed under a Biorad 1024 laser scanning confocal microscope (Nikon X60 oil immersion objective with 1.4 numerical aperture). Elastin was excited at a wavelength of 488 nm, and a band pass filter (506-538 nm) was used to detect fluorescence. CY3 was excited at 568 nm, and fluorescence was detected between 589 and 621 nm.

3.3 Results

3.3.1 Neointimal Thickening in the Rat Carotid after Balloon Catheter Injury

Hematoxylin and Eosin (H&E) cross-sections of control and injured carotids at 1 week, 3 weeks and 8 weeks after injury displayed a gradual thickening of the neointima as time progressed (Figure 3.1). In the uninjured carotids, endothelial cells formed a monolayer in the intima and were oriented longitudinally in the direction of flow. Medial smooth muscle cells were oriented circumferentially between the layers of elastic lamina (Figure 3.1a). At 1 week after injury, neointimal smooth muscle cells were densely packed together and were randomly oriented (Figure 3.1b). At 3 weeks, the neointima was thicker with more separation between cells (Figure 3.1c). By 8 weeks the neointima had reached its maximal thickness (Figure 3.1d).

3.3.2 Phenotypic Modulations of Smooth Muscle Cells

As smooth muscle cells migrated into the neointima they underwent a process of de-differentiation to a synthetic state. This process was evident by the change in shape and orientation of cells combined with the decreased levels of myofilament expression. Medial smooth muscle cells present in the first lamellar unit of an uninjured carotid stained en face with rhodamine phalloidin displayed actin filaments throughout the cells. These cells were thin and elongated and were aligned circumferentially within the vessel (Figure 3.2a). En face neointimal smooth muscle cells at 1 week after injury stained with rhodamine phalloidin displayed actin filaments present around the periphery of the cell but substantially reduced within the cell (Figure 3.2b). Cells were not elongated and
were of various sizes. This decrease in actin filament expression was primarily due to a decrease in the α-isoform of actin at early time points after injury. Cross-sections immunostained to detect smooth muscle-α-actin showed a decrease in the number of filaments staining positive for smooth muscle-α-actin immediately after injury, then a gradual increase. The protein was present throughout the contractile quiescent cells in the media at all time points (Figure 3.3a) but was absent in the neointima at 1 week (Figure 3.3b) even though a neointima populated by smooth muscle cells was present (Figure 3.3b inset). At 3 and 8 weeks smooth muscle-α-actin was localized around the plasma membrane of neointimal smooth muscle cells (Figure 3.3c & 3.3d). Immunoblots probed with an antibody to smooth muscle-α-actin of whole carotid lysates detected a single band at approximately 43kd with an additional band detected at approximately 33 kd. No significant differences were noted in the intensities of the upper molecular weight band across the 3 time points and control carotids however there were fluctuations in the presence and intensity of the 33kd band (Figure 3.4).

### 3.3.3 Neointimal Levels of N-cadherin, β-catenin and Plakoglobin

Levels of N-cadherin, β-catenin and plakoglobin were also changed coincident with the change in phenotype of smooth muscle cells. Cross-sections of control carotids and injured carotids 1 week, 3 weeks and 8 weeks after injury immunostained to detect N-cadherin showed that neointimal levels of N-cadherin increased at 1 and 3 weeks and decreased at 8 weeks after injury back to medial levels (Figure 3.5). Medial and adventitial staining was comparable across all the sections. To ensure that this pattern of staining was not due to non-specific cross-reactivity, cross sections were immunostained with donkey anti-goat CY3 conjugated secondary antibody only (Figure 3.6). Staining was absent except for minimal cross-reactivity within the neointima at 8 weeks. In addition, control carotid cross-sections immunostained with anti-N-cadherin antibody pre-incubated with its blocking peptide (Figure 3.7b) abolished most of the signal compared to N-cadherin antibody alone (Figure 3.7a).

When arterial cross-sections were immunostained to detect β-catenin, neointimal levels of β-catenin were increased at 1 and 3 weeks and decreased at 8 weeks after injury.
β-catenin was present within the media at comparable levels across all time points and adventitial staining was comparable across all the sections (Figure 3.8). Similar findings were seen with plakoglobin immunostained cross-sections with increased neointimal levels at 1 and 3 weeks and decreased levels at 8 weeks. In contrast to the other two antibodies, plakoglobin displayed minimal adventitial staining across all the time points (Figure 3.9).

Immunoblot analysis of whole carotid lysates revealed the same trend as the immunofluorescent staining. The highest levels of all three proteins was detected at 1 week after injury with decreasing levels approaching control (uninjured carotid) by 8 weeks after injury. Blots displayed a single band at 135kd when probed for N-cadherin, a single band at 92kd for β-catenin and a doublet at 82kd for plakoglobin (Figure 3.10). The plakoglobin doublet may have been due to antibody detecting both phosphorylated and dephosphorylated form of the protein.

### 3.3.4 Distribution of Neointimal N-cadherin, Plakoglobin and β-catenin

Neointimal smooth muscle cells 1 week after injury immunostained to detect N-cadherin (Figure 3.11a), β-catenin (Figure 3.11b) and plakoglobin (Figure 3.11c) showed staining localized around the plasma membrane of the cells. Confocal micrographs of single optical sections collected at a separation of 0.40 μm from the luminal (apical) to basal surface of the cell immunostained to detect β-catenin (representative protein of adherens junction plaques) displayed the protein localized around the entire plasma membrane of the cell (Figure 3.12).
Figure 3.1: Light microscope pictures of cross-sections of an uninjured control carotid (A) and carotids at 1 week (B), 3 weeks (C) and 8 weeks (D) after injury stained with H&E. Arrow points to internal elastic lamina. Lumen is toward the top in all four panels. A single layer of endothelial cells is present in the intima in the uninjured control artery (arrowhead). At 1 week after injury, smooth muscle cells are present in the neointima and are tightly packed together. Neointimal thickness increases at 3 weeks with greater separation between cells. Neointimal thickness increases even further by 8 weeks.
Figure 3.2: Confocal micrograph of single optical sections of uninjured medial smooth muscle cells (A) and neointimal smooth muscle cells 1 week after injury (B). Carotid arteries were stained with rhodamine phalloidin and viewed en face. Medial cells are long and narrow with myofilaments throughout the cell. Neointimal cells are rounded with myofilaments localized mainly to the periphery of the cells.
Figure 3.3: Confocal micrograph projections of cross-sections of a control uninjured carotid (A) and carotids at 1 week (B), 3 weeks (C) and 8 weeks (D) after injury immunostained to detect smooth muscle-α-actin (red). Elastin autofluoresces green. Lumen is on the left in all four panels. Smooth muscle-α-actin is present throughout the cells in the media across all the time points. At 1 week after injury, a neointima is present and is populated with smooth muscle cells (insert B-arrow), however they express low levels of smooth muscle-α-actin. By 3 weeks, the protein is expressed around the plasma membrane of neointimal smooth muscle cells and persists at 8 weeks.
Figure 3.4: Immunoblot of whole carotid lysate and smooth muscle cell lysates probed with an antibody to smooth muscle-α-actin. A prominent band at 43 kd is present in all the lanes in equal amounts. A lower molecular weight band (33kd) is present in some of the lanes and varies in intensity.
Figure 3.5: Confocal micrograph projections of cross-sections of a control uninjured carotid (A) and carotids at 1 week (B), 3 week (C) and 8 week (D) after injury immunostained to detect N-cadherin (red). Elastin autofluoresces green. Lumen is on the left in all four panels. N-cadherin is expressed at equal levels within the media across all the time points. Neointimal N-cadherin levels are elevated at 1w and 3 weeks after injury and decreased at 8 weeks.
Figure 3.6: Confocal micrograph projections of cross-sections of a control uninjured carotid (A) and carotids at 1 week (B), 3 week (C) and 8 week (D) after injury immunostained with a CY3 conjugated secondary antibody only (red). Elastin autofluoresces green. Lumen is on the left in all four panels. No staining is present except for minimal cross-reactivity within the neointima at 8 weeks.
Figure 3.7: Confocal micrograph projections of right carotids immunostained with an antibody to N-cadherin (A) and with an antibody to N-cadherin pre-incubated with its blocking peptide (B) (red). Elastin autofluoresces green. Lumen is on the left in both panels. Pre-incubation of the antibody with the blocking peptide abolished the signal that was seen with antibody alone.
Figure 3.8: Confocal micrograph projections of cross-sections of a control uninjured carotid (A) and carotids at 1 week (B), 3 weeks (C) and 8 weeks (D) after injury immunostained to detect β-catenin (red). Elastin autofluoresces green. Lumen is on the left in all four panels. β-catenin is expressed at equal levels within the media across all the time points. Neointimal β-catenin levels are elevated at 1 week and 3 weeks after injury and decreased at 8 weeks.
Figure 3.9: Confocal micrograph projections of cross-sections of a control uninjured carotid (A) and carotids at 1 week (B), 3 weeks (C) and 8 weeks (D) after injury immunostained to detect plakoglobin (red). Elastin autofluoresces green. Lumen is on the left in all four panels. Plakoglobin is expressed at equal levels within the media across all the time points. Neointimal plakoglobin levels are elevated at 1 and 3 weeks and decreased at 8 weeks.
Figure 3.1: Immunoblots of whole carotid and porcine aortic smooth muscle cells lysate probed with antibodies to N-cadherin (A), β-catenin (B) and plakoglobin (C). At 1 week, all three protein levels are highest and gradually decrease to near control levels by 8 weeks.

Figure 3.10: Immunoblots of whole carotid and porcine aortic smooth muscle cells lysate probed with antibodies to N-cadherin (A), β-catenin (B) and plakoglobin (C). At 1 week, all three protein levels are highest and gradually decrease to near control levels by 8 weeks.
Figure 3.11: Confocal micrographs of single optical sections of neointimal smooth muscle cells 1 week after injury. Carotids were immunostained to detect N-cadherin (A), β-catenin (B) and plakoglobin (C) and viewed en face. Proteins are localized around the plasma membrane of smooth muscle cells.
Figure 3.12: Confocal micrographs of optical sections collected at a separation of 0.40 μm from the most luminal surface (top left) to basal surface (bottom right) of neointimal smooth muscle cells 1 week after injury. Carotids were immunostained to detect β-catenin and viewed en face. Cell highlighted by an asterisk has β-catenin localized around the entire plasma membrane.
3.4 Discussion

Neointimal growth after balloon injury in rat carotid arteries was characterized at 1 week by tightly packed smooth muscle cells. At 3 weeks, the neointima was thicker due to continued migration and proliferation and deposition of extracellular matrix constituents. By 8 weeks the thickness had reached its maximum. Biochemical data has shown that both medial and intimal smooth muscle cells remain in a synthetic phenotype until at least 15 days after injury. This is characterized by a shift in the isoform of actin from α (main isoform present in contractile cells) to β (characteristic of a de-differentiated smooth muscle cell). The predominance of the α-isoform returns by 60 days in both medial and intimal smooth muscle cells suggesting that cells have reverted back to a differentiated state (Kocher et al., 1991). My immunofluorescent analysis of cross-sections stained with smooth muscle-α-actin revealed that neointimal protein levels were reduced at 1 week but returned by 3 weeks and persisted in the 8 week time point. These myofilaments were almost exclusively localized around the periphery of the cell. Immunoblots probed with an antibody to smooth muscle α-actin displayed 2 distinct bands at 43kd and 33kd. The 33kd band may represent a proteolytic degradation product of the protein as some blots did not present the lower band while others only displayed it in certain lanes. Overall, there was no observable trend suggesting that the preparation of individual lysates may have caused degradation of the protein. The 43kd band intensity was comparable across all lanes which is at odds with the immunofluorescent data. This may have been due to the large amounts of overall protein loaded and the extremely high affinity of the antibody preventing the detection of subtle differences.

I also found that neointimal smooth muscle cells had a distinct morphology and orientation compared to cells in the media. Cross-sections stained with H & E and medial en face sections stained with rhodamine phalloidin showed that medial cells were oriented circumferentially around the vessel wall and were elongated. In contrast, neointimal smooth muscle cells 1 week after injury stained with rhodamine phalloidin were round and cobblestone with small myofilament bundles located around the periphery of the cell, a characteristic of the synthetic phenotype (Campbell and Campbell, 1987). At 1 week after injury, neointimal smooth muscle cells displayed N-cadherin, β-
catenin and plakoglobin localized around the entire plasma membrane of the neointimal smooth muscle cells.

Cross-sections of carotids immunostained to detect N-cadherin showed that the protein was equally expressed across all medial layers for all the time points. Examination of medial smooth muscle cell distribution of N-cadherin was not possible as the antibody used for en face immunostaining did not penetrate past the internal elastic lamina. Adventitial staining was present in cross-sections immunostained with the N-cadherin, β-catenin and plakoglobin polyclonal antibodies but was eliminated when a monoclonal antibody against plakoglobin was used. Based on this, I inferred that the adventitial staining was most likely an artifact caused by non-specific binding of the polyclonal antibody.

Neointimal levels of N-cadherin and its associated catenins changed over time in the carotid injury model. Immunofluorescence and western blotting showed that the highest levels of N-cadherin in the neointima were at early time points (most significant at 1 week) with levels back to control by 8 weeks. The fluctuation of N-cadherin levels paralleled the temporal migration of smooth muscle cells that occurred in response to injury. The functional significance of these increased levels are at odds with the basic cell migration model in which decreased contact with neighboring cells is associated with cell movement. If, according to this model, N-cadherin adherens junctions were mediating only static cell-cell adhesion, levels would be expected to decrease when cells are actively migrating, however the opposite was seen. Similarly, another protein expressed in vascular smooth muscle cells that is involved in cell-cell communication, connexin-43, is upregulated in the intima at early time points after injury (Yeh et al., 1997). It is reasoned that upregulation of connexin-43 gap junctions between smooth muscle cells allows for increased capacity for direct intercellular signaling contributing to the maintenance of the synthetic phenotype (Yeh et al., 1997).

N-cadherin adherens junction complexes may also be linked to phenotype regulation. Increased neointimal levels of N-cadherin, β-catenin and plakoglobin were detected in the neointima at 1 and 3 weeks after injury. This corresponds to a time in which cells are in a synthetic phenotype and are in the midst of breaking and re-establishing cell-cell contacts. Immunoblot analysis confirmed these findings with the
most intense bands present at 1 week with the intensity dropping off to near control levels by 8 weeks. Immunofluorescent staining at 8 week showed that neointimal intensity of N-cadherin was comparable to medial intensity at the 8 weeks time point when the bulk of cells have returned to a more contractile phenotype.

Based on the results that both plakoglobin and β-catenin levels were down at 8 weeks when the bulk of the neointimal cells have returned to a quiescent state refutes my original hypothesis of preferential association of plakoglobin versus β-catenin as cells reverted back to a contractile phenotype. Instead it appears that the proteins both underwent coincident modulation as the cells migrated and modulated their phenotype. In conclusion, adherens junctions in smooth muscle cells consisting of N-cadherin, β-catenin and plakoglobin are dynamic structures that change in relation to the state of the cell. These proteins are upregulated at early time points after injury a time at which cells are undergoing active migration and decreased at later time points when cells are more quiescent and stable. The functional significance of the increase in N-cadherin adherens junction proteins may be to promote phenotypic modulation and/or migration.
CHAPTER 4
General Discussion and Future Experiments
4.1 General Discussion

Cultured porcine aortic smooth muscle cells were found to express adherens junction plaques composed of N-cadherin, α-catenin, β-catenin and plakoglobin localized at the basolateral extensions of contacting cells. Wounded cultured smooth muscle cells pre-incubated with a function blocking antibody against N-cadherin displayed reduced processes into the wound area and a decreased rate of wound closure compared to control cells pre-incubated with mouse IgG. These results suggest that N-cadherin adherens junctions may promote smooth muscle cell migration. N-cadherin has been proposed to be capable of mediating both labile and stable cellular interactions that facilitate dynamic cell processes such as cell migration and neurite outgrowth (Hatta et al., 1997; Bixby and Zhang, 1990; Riehl et al., 1996). In my migration model, N-cadherin adherens junctions may be facilitating the movement of smooth muscle cells by virtue of its capacity to mediate transient contacts. If adhesion between N-cadherin complexes is blocked, smooth muscle cells can no longer navigate past one another which results in more blunted processes and reduced migration. As an adjunct to this model, N-cadherin adherens junction complexes through their stabilizing links to the cytoskeleton may allow force to be transmitted between adjacent cells enabling them to move past one another. In fibroblasts, viscoelastic tension was shown to be transmitted directly from cell to cell mediated by the mechanical coupling of N-cadherin adherens junctions (Ragsdale et al., 1997).

The involvement of N-cadherin with smooth muscle cell migration was further investigated using an in vivo denuding injury. Neointimal smooth muscle cells had increased levels of N-cadherin, β-catenin and plakoglobin at 1 and 3 weeks after injury with decreased levels at 8 weeks. These fluctuations of N-cadherin adherens junction proteins paralleled the phenotypic modulation of smooth muscle cells as well as smooth muscle migration into the intima. Functionally, early increases in N-cadherin within neointimal smooth muscle cells may be important for migration by promoting a state of dynamic adhesion that allows both attachment and detachment of cells. At later time points, neointimal levels of N-cadherin were comparable to control medial levels. This decrease may be related to the state of the cells and their contacts. At approximately 8
weeks after injury, the bulk of the smooth muscle cells have reverted back to a more contractile phenotype and have re-established more stable junctions with extracellular matrix constituents. Cells are no longer migrating and therefore transient cell-cell contacts mediated by N-cadherin are no longer needed and may be downregulated.

In addition to the structural role that N-cadherin may be playing in migration, it may also be implicated in a migratory signaling pathway. Other cell types undergoing dynamic processes employ N-cadherin in signaling pathways involved in controlling these events. Neurite outgrowth has been shown to be dependent on N-cadherin binding to and activating the FGF receptor independently of ligand binding and promoting downstream signaling (Doherty et al., 1991; Lom et al., 1998). Similarly, in a cancer cell line, enhanced migration and production of MMP-9 was noted when cells were treated with FGF-2. Interestingly, only those cells that expressed N-cadherin displayed these results (Hazan et al., 2000). In the rat injury model, many growth factors are released, including FGF. It is possible that the increased levels of N-cadherin on the surface of smooth muscle cells at early time points after injury may bind to the FGF receptor and further upregulate the signaling pathway leading to enhanced migration. When cells revert back to a more contractile phenotype, N-cadherin levels are decreased from the cell surface decreasing the activation of the FGF receptor and its downstream signals that promote migration.

Migration involves a multitude of factors and molecules that must be precisely regulated. These experiments have shown that N-cadherin mediates cell-cell adhesion in vascular smooth muscle cells. These junctions were shown to be important in the migration of smooth muscle cells and that the levels are dynamically regulated as cells undergo phenotypic modulation and migrate. Future studies will need to dissect the precise function of N-cadherin in migration and if it is linked to the change in phenotype of smooth muscle cells.
4.2 Future Experiments

1) In vivo immunofluorescent staining and western analysis results showed that N-cadherin is upregulated at early time points after injury. To further investigate the temporal regulation of N-cadherin involved in smooth muscle cell migration, shorter time points will be used to determine how soon after injury N-cadherin is upregulated and when the actual peak of protein expression is.

2) Balloon injured vessels displayed a gradual decrease in the levels of N-cadherin in the neointima, most significantly at 8 weeks. Immunoblots probed for N-cadherin did not display an extra lower molecular weight band at the 8 week time point suggesting that N-cadherin is not proteolytically degraded into its extracellular domain (97kd) and its cytoplasmic and transmembrane domains. To determine if N-cadherin is excluded from the adherens junctions in addition to being downregulated at later time points western analysis of whole carotid lysates comparing the triton soluble and triton insoluble fractions at the 3 time points will be probed for N-cadherin.

3) N-cadherin has been shown to associate with FGF receptor and promote activation of this receptor leading to outgrowth of neurites (Williams et al., 1994). To determine if this signaling pathway is activated in the migration of smooth muscle cells, in vitro wound experiments will be performed. Wounded cultures of smooth muscle cells will be analysed using western blotting to determine if FGF receptor is activated/phosphorylated when N-cadherin is not inhibited and if the phosphorylated levels of FGF receptors decrease when N-cadherin is inhibited with the blocking antibody.

4) Cross-talk between integrins and N-cadherin levels has been implicated in the regulation of migration in neural crest cells and myoblasts (Monier-Gavelle and Duband, 1997; Huttenlocher et al., 1998). To determine if certain integrins are involved in the regulation of N-cadherin during migration, blocking antibodies against different integrin subunits will be administered to wounded cultured porcine aortic smooth muscle cells. Wound edge growth will be measured and western blots of smooth muscle cell lysates
will be probed for N-cadherin at different time points after injury to determine if levels are modulated.
References


APPENDIX A

Smooth Muscle-Endothelial Cell Interactions Mediated by N-Cadherin

Introduction

In the uninjured vessel wall endothelial cells and smooth muscle cells are separated by their respective basement membranes and an internal elastic lamina that is highly fenestrated. Studies measuring dye and current movements have shown that electrical and chemical coupling exists between smooth muscle cells and endothelial cells (von der Weid et al., 1993; Little et al., 1995). Physical evidence of these junctions was shown through the discovery of tiny pentalaminar gap junctions (Sandow and Hill, 1999). Smooth muscle cells and endothelial cells also directly interact with each other under pathological conditions such as during re-endothelialization after denuding injury. In these vessels, endothelial cells either directly overlie or laterally oppose the luminal smooth muscle cells and form regions of focal membrane contacts (Kocher et al., 1995). In vitro, experiments using co-cultures of smooth muscle cells and endothelial cells separated by a porous membrane have shown that smooth muscle cells can extend cytoplasmic projections through the pores to make intimate contact with endothelial cells (Fillinger et al., 1997). These interactions may be mediated by cadherin complexes.

In vitro, adhesion between smooth muscle cells and endothelial cells has been shown to be calcium dependent, a characteristic of adhesion mediated by cadherins (Gilbertson-Beadling and Fisher, 1997). In vivo, N-cadherin was found in smooth muscle cells present in the most luminal medial layer and in the endothelial cells lining the intima (Gilbertson-Beadling and Fisher, 1997). Although cadherins normally mediate only homotypic and homologous contacts there are some cells in which cadherins mediate homotypic and heterologous contact (Sandig et al., 1997). Based on the results from these studies I hypothesized that endothelial cells and smooth muscle cells make heterologous contact mediated in part by N-cadherin.
Material and Methods

En face Immunostaining

Uninjured carotids were perfusion fixed with 3% paraformaldehyde for 4 minutes then rinsed with PBS. The adventitia was cleared off and arteries were opened longitudinally and pinned flat with the intimal layer facing up. Arteries were incubated with 0.2% Triton X-100 for 5 minutes then rinsed 3x5 minutes with PBS containing Mg2+ and Ca2+. Arteries were incubated with the following primary antibodies for 1 hour: goat polyclonal anti-β-catenin antibody 1:50 (Santa Cruz), goat polyclonal anti-N-cadherin 1:50 (Santa cruz), goat polyclonal anti-plakoglobin antibody 1:50 (Santa Cruz), mouse monoclonal anti-connexin-43 1:50 (Transduction Laboratories) or mouse monoclonal anti-paxillin 1:50 (Transduction Laboratories). Arteries were rinsed with PBS 3x5 minutes then incubated with a CY3 conjugated donkey anti-goat antibody (Jackson Immuno Research Laboratories) in a dilution of 1:100 for 30 minutes in the dark. Following incubation, the arteries were rinsed 3x5 minutes with PBS. Whole mount preparations with intima side up were cover slipped with glycerol:PBS 9:1 and viewed under a Biorad 1024 laser scanning confocal microscope (Nikon X60 oil immersion objective with 1.4 numerical aperture). Elastin was excited at a wavelength of 488 nm, and a band pass filter (506-538 nm) was used to detect fluorescence. CY3 was excited at 568 nm, and fluorescence was detected between 589 and 621 nm.

Immunostaining of Cultured Cells

Porcine aortic smooth muscle cells and endothelial cells between passages 4 and 8 were plated on glass coverslips (22 x 22mm) in medium 199 with 5% FBS, 1% fungazone and 1% penicillin streptomycin in a 37°C incubator with 5% CO2. Cells were rinsed with PBS containing Ca2+ and Mg2+ 3x5 minutes then fixed with 3% paraformaldehyde for 20 minutes. Cells were rinsed with PBS 3x5 minutes then incubated with 0.2% Triton X-100 for 5 minutes. The cells were rinsed with PBS 3x5 minutes then incubated with mouse monoclonal anti-N-cadherin antibody (Transduction Laboratories) in a dilution of 1:50 or mouse monoclonal anti-VE-cadherin antibody (ICOS Laboratories) in a dilution of 1:50 for 1 hour. Cells were rinsed with PBS 3x5 minutes then incubated with an FITC conjugated donkey anti-mouse antibody (Jackson
ImmunoResearch Laboratories) in a dilution of 1:50 for 30 minutes in the dark. Some cells were co-incubated with rhodamine phalloidin 1:20. The coverslips were then rinsed with PBS 3x5 minutes, mounted on glass microscope slides with glycerol:PBS (9:1) and viewed under a Biorad 1024 laser scanning confocal microscope (Nikon X60 oil immersion objective with 1.4 numerical aperture). FITC was excited at a wavelength of 488 nm, and a band pass filter (506-538 nm) was used to detect fluorescence. CY3 was excited at 568 nm, and fluorescence was detected between 589 and 621 nm.

**Direct Co-Cultures**

Porcine aortic endothelial cells were grown to various percentages of confluency (50%, 80%, & 100%) on glass coverslips (22 x 22 mm) in medium 199 with 5% FBS, 1% fungazone and 1% penicillin streptomycin in a 37°C incubator with 5% CO₂ after which porcine aortic smooth muscle cells were plated at various densities (3.0 x 10⁵ cells, 1.5 x 10⁶ cells & 7.5 x 10⁶ cells). 2 days after plating the smooth muscle cells, the co-cultures were stained for N-cadherin as per the immunostaining of cultured cells protocol described above.

**Membrane Co-Cultures**

1.0 x 10⁵ cells of primary porcine aortic endothelial cells were plated onto the outer surface of a 13μm thick, 25 mm diameter polyethylene terephthalate (PET) membrane with 1.0μm pores configured at a density of 1.6 million pores/cm² (Cyclopore membrane, Falcon cell culture insert, Becton Dickinson). After endothelial cells reached confluency (2-3 days), smooth muscle cells were plated at 5.0 x 10⁴ cells per well on the inner surface of the membrane opposite the previously seeded endothelial cells. Cells used for co-cultures were between passage 4-8 and were grown in medium 199 with 5% FBS, 1% fungazone and 1% penicillin streptomycin. 10 days after plating the smooth muscle cells, the membranes were immunostained with rhodamine phalloidin and N-cadherin as per the immunostaining of cultured cells protocol described above.
Results

Junctional Proteins in Vascular Endothelial Cells

Intimal monolayers of endothelial cells express gap junctions mediated largely by connexin-43. Staining for this protein was evident at the borders between contacting cells in a fine chain-like pattern (Figure A.1a). At the level of the internal elastic lamina there were some areas of positive staining for connexin-43 were localized to fenestrae (Figure A.1b). Endothelial cells also express paxillin, a protein involved in the focal adhesion complexes that mediate basal interactions with the extracellular matrix. This protein was distributed throughout the cell in dashes lined up along the length of the cell (Figure A.2a). At the level of the internal elastic lamina, the protein was diffusely localized at the areas of the fenestrae (Figure A.2b). N-cadherin staining was diffuse within the endothelial monolayer and was distinctly absent from lateral cell contacts (Figure A.3a). At the level of the internal elastic lamina, N-cadherin was punctately localized to the fenestrae (Figure A.3b). Control sections stained with anti-N-cadherin antibody incubated with its blocking peptide (Figure A.4a) and with secondary fluorophore conjugated antibody only (Figure A.4b) showed minimal staining in the intima. The punctate localization of N-cadherin within the fenestrae suggested that perhaps adherens plaques were present between endothelial cells and underlying smooth muscle cells. To confirm this possibility immunostaining was done using an antibody that detected one of the catenin proteins found in adherens junctions in endothelial cells, β-catenin. Endothelial cells displayed strong staining for this protein with its distribution localized at the cell-cell junctions (Figure A.5a). At the level of the internal elastic lamina there were some regions where the protein was present at the fenestrae (Figure A.5b) however these areas corresponded to adherens junctions mediated by VE-cadherin and not N-cadherin (data not shown).

Lack of N-cadherin Localization Between Contacting Endothelial and Smooth Muscle Cell Co-Cultures

Endothelial cells stained with an antibody to detect N-cadherin (Figure A.6a) showed diffuse staining of the protein throughout the plasma membrane. In contrast, cells stained with an antibody to detect VE-cadherin (Figure A.6b) displayed intense VE-
cadherin localization around the midplane of the plasma membrane of the cell mediating contact with other cells in the monolayer. In the direct co-culture systems 2 days after plating smooth muscle cells onto an endothelial monolayer there was evidence of adherens junction formation between contacting smooth muscle cells (Figure A.7a), however there were no localized plaques consisting of N-cadherin between endothelial cell and smooth muscle cells (Figure A.7b,c). Confocal vertical sections of co-cultures grown on porous PET membranes stained with rhodamine phalloidin showed the presence of smooth muscle cells on the top side of the membrane separated from endothelial cells on the underside of the membrane by a 13 μm porous membrane. After 9 days of growth, membranes with 0.45 μm pores showed no processes of either cell type extending through the membrane (fig A.8a). At this time point endothelial cells had been plated on the underlying surface of the membrane for approximately 14 days and had lost their normal cobblestone appearance and had acquired a more elongated spindle phenotype (data not shown). In contrast, 9 days after plating smooth muscle cells alone on the top of a membrane with 1.0 μm pores exhibited numerous extensions, however only a few that extended through the entire width of the membrane (Figure A.8b).
Figure A.1: Confocal micrographs of single optical sections of endothelial cells at the midplane of the intima (A) and at the internal elastic lamina. Carotid arteries were immunostained to detect connexin43 (red) and viewed *en face*. Elastin autofluoresces green. Connexin 43 is localized to endothelial plasma membranes in a chain-like pattern. Connexin43 is also localized to the fenestrae of the internal elastic lamina.
Figure A.2: Confocal micrographs of single optical sections of endothelial cells at the midplane of the intima (A) and at the internal elastic lamina (B). Carotid arteries were immunostained to detect paxillin (red) and viewed en face. Elastin autofluoresces green. Paxillin is distributed towards the basal surface of the cell in a dashed pattern that is aligned with the long axis of the cell. Paxillin is diffusely localized to the fenestrae of the internal elastic lamina.
Figure A.3: Confocal micrograph projection of single optical sections collected at a separation of 0.25 μm from the luminal to basal surface of the endothelium (A). Confocal micrograph of a single optical section at the internal elastic lamina (B). Carotid arteries were immunostained to detect N-cadherin (red) and viewed en face. Elastin autofluoresces green. N-cadherin was diffuse localized around the plasma membrane of endothelial cells and was absent from contacts between cells. At the level of the internal elastic lamina, N-cadherin was punctately localized at the fenestrae.
Figure A.4: Confocal micrographs of single optical sections of endothelial cells at the midplane of the intima. Carotid arteries were immunostained with N-cadherin pre-incubated with its blocking peptide (A) and a CY3 conjugated secondary antibody only (B). Minimal staining was present in both controls.
Figure A.5: Confocal micrographs of single optical sections of endothelial cells at the midplane of the intima (A) and at the internal elastic lamina. Carotid arteries were immunostained to detect β-catenin (red) and viewed en face. Elastin autofluoresces green. β-catenin is localized to cell-cell contacts outlining the shape of the cell. At the level of the internal elastic lamina there are some regions where the protein is localized to the fenestrae.
Figure A.6: Confocal micrographs of porcine aortic endothelial cells immunostained to detect N-cadherin (A) and VE-cadherin (B). The images are projections of optical sections collected at a separation of 0.25 μm from the apical to basal surface of the cells. N-cadherin is diffusely localized around the plasma membrane of the cells while VE-cadherin is concentrated at cell-cell contacts.
Figure A.7: Confocal micrographs of porcine aortic smooth muscle cells grown for 2 days on a confluent monolayer of porcine aortic endothelial cells. Cells are immunostained to detect N-cadherin. The images are projections of optical sections collected at a separation of 0.25 mm from the apical to basal surface of the cells. N-cadherin localizes to smooth muscle-smooth muscle contacts and mediates adhesion but remains diffuse over the cell membrane at smooth muscle-endothelial cell contacts (B,C). Endothelial cells identified by asterisks.
Figure A.8: Confocal vertical micrograph projections of smooth muscle/endothelial co-cultures grown on PET membranes stained with rhodamine phalloidin. Membrane containing 0.45 μm pores with SMCs on top and ECs on bottom (A). Membrane containing 1.0 μm pores with SMCs on top only (B). Arrow denotes part of the SMC process that has extended through the entire width of membrane. After 9 days of growth membranes with 0.45 μm pores showed no processes of either cell type extending through the membrane. In contrast, 9 days after plating smooth muscle cells alone on the top of a membrane with 1.0 μm pores exhibited some extensions, however only a few that extended through the entire width of the membrane.
Discussion

In vivo immunofluorescent staining localized several junctional proteins to the fenestrae of the internal elastic lamina, an area of possible contact between endothelial cells and smooth muscle cells. Connexin-43 decorated the periphery of endothelial cells in a punctate pattern with areas of staining that were localized to the fenestrae. Likewise N-cadherin displayed strong punctate staining at the fenestrae even though overall the pattern was relatively diffuse across the plasma membrane of the endothelial cell. The punctate distribution of these two proteins at the internal elastic lamina suggested that they might be involved in mediating interactions between endothelial cells and smooth muscle cells. Gap junctions have been reported to exist between these two cell types allowing passage of electrical and metabolic signals (Blennerhassett et al., 1987). Cadherin interactions may be mediating other signals between the two cell types. In order for cadherins to form functional adherens junctions, the cytoplasmic domain must be linked to the catenin proteins. To further investigate whether N-cadherin adherens junctions were present at fenestrae, tissue was stained for β-catenin. Unlike N-cadherin, this protein was distinctly localized in a continuous ring around the borders of contacting cells. Although at the level of the internal elastic lamina this protein occasionally localized to fenestrae these areas of β-catenin staining did not correspond to areas of positive N-cadherin staining. These results suggest that N-cadherin is not mediating junctions between endothelial cells and smooth muscle cells. Alternatively, the N-cadherin adherens junction plaques may be too small to be detected through immunofluorescence.

Cultured endothelial cells express both N-cadherin and VE-cadherin however their localization is very different. N-cadherin is diffuse throughout the cell membrane while VE-cadherin strongly outlines the plasma membrane at the midplane of the cell. N-cadherin did not preferentially localize to areas of endothelial-smooth muscle cell contacts in the direct co-culture system used. The lack of staining between endothelial and smooth muscle cells may have been because N-cadherin is not involved in mediating this type of interaction or that it is not involved in this process for porcine cells. Alternatively, it may be that this method was not conducive for the appearance of these junctions. Co-culture systems using a porous membrane that allowed for contact
between cell types grown on either side of the membrane but prevented migration were used to reproduce the in vivo spatial orientation of cells. Membranes with 0.45 \( \mu \text{m} \) pores had very few smooth muscle cell extensions into the pores even up to 14 days after plating (data not shown). This could have been due to the small pore size or that endothelial cells were releasing factors such as soluble heparin that inhibited smooth muscle cell growth. In addition, after 9 days, cultured endothelial cells were no longer cobblestone in appearance and the layer was characterized by overgrowth of long spindle shaped cells. This may have been due to contamination and overgrowth by another cell type present in the primary culture or a change in phenotype of the existing endothelial cells. When smooth muscle cells were plated alone on membrane with 1.0\( \mu \text{m} \) pores there were only a few extensions that extended the width of the membrane. I concluded that the optimal plating time to maintain a normal phenotype of cells on the membrane was too short to enable long, numerous smooth muscle cell extensions through the entire width of the membrane allowing contact with the underlying endothelial cells.

Direct evidence of N-cadherin mediated contact between endothelial and smooth muscle cells was not found with these in vitro experiments however it does not eliminate the possibility that these junctions exist and are biologically relevant. The membrane co-culture system continues to be an attractive way of qualitatively localizing endothelial and smooth muscle cell contact. In order to achieve results with this system, cells need to maintain a normal morphology and need to be able to extend lengthy processes into the pores of the membrane. Primary cultures did not display these characteristics. An alternative may be to use endothelial and smooth muscle cell lines. In general, these cultures are more stable and exhibit specific well established characteristics of morphology, migration and proliferation thus removing some of the variability seen with primary cultures.
APPENDIX B
Double Ligation Injury and Organ Culture

Materials and Methods

Double Ligation Injury

Male Sprague-Dawley rats 350-400g were anesthetized with an intraperitoneal 0.70 ml injection of xylazine (3.2mg/ml) and ketamine (49mg/ml). A midline incision was made in the neck. An osmotic minipump (2ML Alzet; 10µl/hour for 7 days) was inserted subcutaneously at the back of the neck. The left external carotid was dissected free of surrounding tissue. A tie was placed downstream of the medial branch of the external carotid and a loose tie was placed just upstream up the medial branch of the external carotid downstream of the carotid bifurcation. The internal carotid was tied off and the common carotid was tied off at the most upstream end. Two small holes were made in the vessel, one in the common carotid just distal to the tie and the other in the external carotid. Blood was flushed out of the vessel using saline followed by the insertion of the pump catheter in the common carotid hole. The artery was then filled with either 50µg/ml of mouse IgG (Sigma) or 50µg/ml of monoclonal anti-N-cadherin antibody (A-CAM from Sigma) solution through the hole in the external carotid that was then tied off. Rats were killed using the euthanasia solution of T61, 1ml/rat, 7 days after injury. 5 control and 5 experimental rats were operated on. Vessels were fixed with 3% paraformaldehyde for 4 minutes at 100mmHg, embedded in paraffin, cut into 5µm sections then stained with H & E.

Organ Culture

Male Sprague-Dawley rats 350-400g were anesthetized with an intraperitoneal 0.70 ml injection of xylazine (3.2mg/ml) and ketamine (49mg/ml). A midline incision was made in the neck and the left external carotid was dissected free of surrounding tissue. A 2F balloon tipped Fogarty catheter was introduced into the left external carotid artery, inflated and passed three times through the common carotid. Rats were killed using the euthanasia solution of T61, 1ml/rat, 7 days after injury. Left carotids were harvested and rinsed several times in PBS containing 2% fungazone and 2% penicillin...
streptomycin. Arteries were cut into 2-3mm rings, placed in media 199 with 5% FBS, 2% fungazone and 2% penicillin streptomycin and incubated at 37°C with 5% CO₂. Arteries were incubated with 10 μg/ml of monoclonal anti-N-cadherin antibody (A-CAM from Sigma) or 10 μg/ml of mouse IgG. Fresh media and antibodies were added every other day. 500 μM of BrdU stock solution was added to each dish 24 hours before fixing (0, 3 and 6 days) at a final concentration of 25 μM. Vessels were rinsed a few times with PBS, fixed for 20 minutes in 3% paraformaldehyde, embedded in paraffin and cut into sections. These sections were deparaffinized in xylene and rehydrated using gradients of ethanol then stained using a biotinylated monoclonal anti-BrdU antibody, counterstained with hematoxylin (Oncogene Research Products) and visualized using streptavidin-peroxidase and diaminobenzindine (DAB).

Results

Morphology of Double-Ligated Carotids

Cross-sections of double ligated carotids stained with H&E showed no noticeable differences between incubation with mouse IgG or N-cadherin function blocking antibody (Figure B.1a,b respectively). There were no visible smooth muscle cells present in the intima 7 days after injury and in many cases the first medial layer was greatly enlarged with reduced number of smooth muscle cells present compared to the other medial layers (Figure B.1 - arrowheads). Inflammatory cells were visible in the adventitia with some infiltration into the media and intima (arrows). Some vessels also displayed a thrombus that partially occluded the vessel lumen (asterisk).

Morphology of Rat Carotids in Organ Culture

Neointimal lesions of injured carotid arteries displayed a significant change in the overall morphology of neointimal lesions after being placed in organ culture. At 1 day, the neointima was comprised of a large number of tightly packed smooth muscle cells (Figure B.2b), by 4 days the cells in the neointima appeared spindle shaped with fewer cells that were greatly separated from one another (Figure B.2c). After 7 days, there were very few cells present and the neointima had substantially decreased in size (Figure
B.2d). The morphology of the neointimas showed no differences between the carotids incubated with N-cadherin antibody or with mouse IgG therefore only N-cadherin antibody pictures were included. The intima of the control carotid remained constant with no intimal growth at all times in organ culture (Figure B.2a is a representative phase contrast picture of all 3 time points).

**Proliferation of Neointimal Cells in Organ Culture**

BrdU staining of cross-sections of injured carotids 1, 4 and 7 days after being placed in organ culture showed that even though the neointima seemed to be decreasing in size, proliferation of neointimal smooth muscle cells was still occurring. BrdU staining in the control carotids remained constant (Figure B.3a is a representative phase contrast picture of all 3 time points). Injured carotids showed no differences in the number of positive BrdU cells between N-cadherin antibody incubation or mouse IgG therefore only N-cadherin antibody pictures are included. Injured carotids had positively stained cells in the neointima at day 1 (Figure B.3b). The number of BrdU positive cells were fewer by 4 days (Figure B.3c) and even less at 7 days (Figure B.3d) compared to day 1. Medial and adventitial staining of BrdU was also evident at 1, 4 and 7 days.
Figure B.1: Light microscope pictures of cross-sections of double ligated carotids after 7 days of incubation with an N-cadherin antibody (Ai, Aii) or mouse IgG (Bi, Bii). No smooth muscle cells are present in the neointima of either group. Inflammatory cells are visible in the adventitia and medial layers of some carotids (arrows). The first medial layer of most carotids have reduced numbers of cells and are enlarged (arrowheads). Some vessels also display an organized thrombus that partially occludes the lumen (asterisk).
Figure B.2: Light microscope pictures of cross-sections of a control uninjured carotid (A) and 1 week injured carotids placed in organ culture after 1 day (B), 4 days (C), and 7 days (D) incubated with an N-cadherin antibody. Carotids were stained with H&E. Uninjured carotids maintained a normal morphology both in the media and the intima over the 7 days. Panel A is a representative picture. In the injured carotids, smooth muscle cells in the neointima at day 1 were tightly packed together. By day 4, cells were fewer in number, more separated and spindle shaped. After 7 days, the neointima had regressed even further.
Figure B.3: Light Microscope pictures of cross-sections of a control uninjured carotid (A) and 1 week injured carotids placed in organ culture after 1 day (B), 4 days (C), and 7 days (D) incubated with an N-cadherin antibody. Carotids were immunostained to detect BrdU. Uninjured carotids had minimal BrdU staining over the 7 days. Panel A is a representative picture. The number of positive BrdU smooth muscle cells present in the neointima of the injured carotids decreased with increasing time in organ culture.
Discussion

The organ culture experiment aimed to inhibit smooth muscle cell migration into the neointima with the administration of a blocking antibody against N-cadherin did not yield the desired results. Similar to other papers published on organ culture (Carere et al., 1992; Dale et al., 1998; Voisard et al., 1999; Holt et al., 1992) the neointimas of rat carotids subjected to balloon catheter injury then maintained in organ culture for 7 days were morphologically different than those in vivo. This difference in neointimal formation may have been due to the lack of physiological flow and pressure that are normally present in vivo. With increasing time in culture neointimal cell number decreased and cells were more spindle shaped and further separated from one another. Although the morphology of the neointima in organ culture suggested a decaying neointima there was still evidence of proliferation through positive BrdU staining. The ability of cells to continue replicating suggests a viable artery, however the number of positively labelled cells did gradually diminish with time in culture. To prevent this altered morphology of the neointima the original protocol was modified slightly. Balloon injured carotids were incubated with higher serum concentrations (20%) and were opened longitudinally and pinned flat instead of floating as rings in the media. These changes did not affect the formation of the altered neointima.

Aside from the altered morphology of the neointima in organ culture, there were no noticeable differences in the overall size of the neointima or proliferation of smooth muscle cells between the arteries incubated with mouse IgG or N-cadherin antibody. This may have been due to the antibody’s inability to enter the neointima and effectively block the N-cadherin mediated binding. Alternatively, the antibody may have been effective in blocking N-cadherin mediated cell adhesion, but the increased levels of N-cadherin in the developing neointima may only be critical at early time points. At 1 week after injury, even though the high levels of N-cadherin may be blocked there may be other pathways that are able to stimulate the neointimal migration.

Inhibition of neointimal smooth muscle cell migration in vivo was attempted through the use of a double ligation model in combination with antibody administration. The double ligation model of rat common carotid arteries is known to cause smooth muscle cell migration and proliferation in the intima without prior injury (Bhawan et al.,
In my experiments, carotids harvested 7 days after double ligation had substantially remodeled and were shorter in length with substantial tissue fibrosis at the site of the inserted catheter. Cross-sections of arteries perfused with N-cadherin function blocking antibody or mouse IgG stained with H&E showed no evidence of smooth muscle cells in the intima 7 days after surgery. Minimal surgical manipulations were performed to minimize the trauma imposed on the vessel and prevent a large inflammatory response, however inflammatory cells were present in the adventitia, media and intima. In many of the vessels examined there appeared to be a severe reduction in number of smooth muscle cells present in the first medial layer as well as an increased separation between the first two medial layers. Some vessels also displayed an organized thrombus which partially occluded the lumen. The lack of smooth muscle cell migration at 7 days after injury regardless of the solution perfused through the carotid suggests that the time course of intimal migration may be longer than reported in other studies using double ligation (Bhawan et al., 1977; Guyton and Karnovsky, 1979). However, based on my results longer periods of time may not lead to intimal thickening but instead result in further progression of an organized thrombus, increased infiltration of inflammatory cells and more medial cell death.

Based on the altered morphology of the neointima in organ culture and the inability to promote smooth muscle cell migration in the double ligated carotid model with or without ACAM incubation, it remains inconclusive as to whether blocking N-cadherin adhesion inhibits the migration of smooth muscle cells into the neointima in vivo.
References


