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The Role of Osteopontin in Vascular Remodeling

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THE ROLE OF OSTEOPONTIN IN VASCULAR REMODELING

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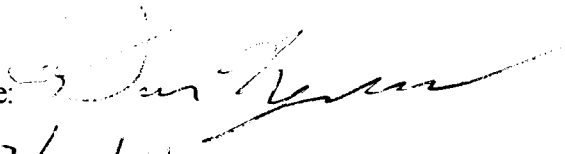
Signature: 

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An Abstract of the Thesis Presented
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Chronic obstructive vascular disease is a major contributor to mortality in developed nations. The pathology typically involves neointima formation accompanied by vascular remodeling in the presence of inflammation. The disease can be modeled in animals with the response to a direct injury of the vasculature. This text describes a project examining the role of osteopontin (OPN) in vascular remodeling. The projectís goal was to mechanistically describe the contribution of OPN to the vascular injury response. The hypothesis that the presence of OPN affects the formation of neointima and constrictive remodeling associated with vascular injury was addressed using the strategy of comparing the remodeling response induced by carotid artery ligation of wild type mice to mice with a null mutation in the geen encoding OPN at time points known to be critical to lesion development.

OPN null mutant mice were compared to wild type mice before and after carotid artery ligation. As substantial variation is observed in the response to carotid artery ligation, the variance was analyzed for potential contributing variables. Distance from the site of ligation was shown to be a significant predictor, and data was analyzed using curvilinear regression. Prior to ligation, OPN null mice had increased heart rate, lower

blood pressure, and increased circulating lymphocytes compared to wild type mice. OPN null vessels also demonstrated greater compliance accompanied by a loosely organized collagen network. Following carotid artery ligation, significant differences were also found in the remodeling response of OPN null animals. At 4 days after ligation, leukocyte adhesion/invasion was diminished by 10-fold in OPN null mice compared to wild type. At 14 days following ligation, the ligated arteries of OPN null mice had smaller neointimal lesions, but greater constrictive remodeling compared to wild type mice, resulting in similar lumen area. Continued remodeling resulted in a similar morphological phenotype in both groups at 28 days.

These data show that endogenous OPN regulates normal vascular physiology, and contributes to the vascular remodeling response by regulating vascular compliance and the inflammatory response.

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OPN	Osteopontin
mRNA	Messenger ribonucleic acid

Chapter 1

THE INVESTIGATION OF CHRONIC OBSTRUCTIVE VASCULAR DISEASE

Cardiovascular disease is the leading cause of mortality in developed nations[1]. According to the World Health Organization, cardiovascular disease accounted for 16,585,000 deaths in the year 2002 or 29.3% of all deaths[2]. In the United States, the American Heart Association reported cardiovascular disease accounts for 945,836 deaths or 39.4% of all deaths for the year 2003. Though the contribution of cardiovascular disease to mortality remains high, advances in the medical treatment of cardiovascular disease have markedly improved outcomes. From 1990 to 2000, the death rate from cardiovascular disease declined 17% in the United States. During this time, medical management of cardiovascular disease improved with the introduction of cholesterol lowering drugs, and vascular surgery methods improved with the introduction of stents. With regard to vascular research, it was less than ten years ago that Thayer et al. described the study of the molecular biology of vascular development as being in the earliest stages[4]. Since then, several of the key interactions involved with the development of the vasculature have been identified. Today, as research continues to investigate the basic functioning of the vasculature, we can expect discoveries that will lead to improved therapies in the future.

Chronic obstructive vascular disease, the major contributor to cardiovascular disease, is a group of distinct pathologies characterized by local changes in the vasculature that result in the restriction of blood flow. The maladaptation causes decreased oxygenation of downstream tissues, and, in some pathology, may result in embolism. As is often the case, if the site of the disease is upstream of a vital area, then progression of the disease may result in death due to failure of that vital area (e.g. stroke, myocardial infarction, etc.). This text presents background information on the pathology common to

all chronic obstructive vascular disease and some recent research into the processes underlying the disease.

Factors affecting cardiovascular function

It is necessary to consider the normal functions of vasculature before examining specific aspects of chronic obstructive vascular pathology. In essence, the mammalian circulatory system is an intermittent pump that circulates fluid through a hierarchical closed system of pipes. Though conceptually simple, the mammalian circulatory system is difficult to characterize due to the variables that affect each of its components[5]. Much of the function of the cardiovascular system can be explained with Poiseuilleís law[5]:

$$Q = (P_1 - P_2)r^4 / 8 n L$$

Where Q is flow rate, P_1-P_2 is the pressure difference, r is the radius of the tube, n is the viscosity of the liquid, and L is the length of the tube.

Poiseuilleís law relates the movement of a fluid through a rigid pipe of known diameter to the fluidís viscosity in response to a pressure drop. The law serves as a nice starting point to examine cardiovascular function as it specifies variables involved in simple fluid flow. However, Poiseuilleís law has two major shortcomings in its ability to describe blood flow in the circulatory system. The law assumes constant pressure in a rigid tube, whereas flow through the vasculature involves pulsatile pressures in an extensible tube. The pulsatile changes in pressure and extensibility of the tube confound the mathematical

description of blood flow such that no expression has been proposed to describe the system. Rather mathematical description is done by analogy to electrical systems (Ohms law: Voltage = amperage x resistance) and series of pumps (windkessel). Thus, experimental analysis of hemodynamics remains largely descriptive while efforts continue to verify the mathematical relations of the variables involved.

The blood

For the purposes of this study, the simplest component of the circulatory system to consider is the blood. Viscosity, defined as shear stress (the force resisting blood flow) divided by shear strain (the change in flow with distance measured perpendicular to the flow), is the resistance of a fluid to change shape. According to Poiseuille, viscosity affects the rate of blood flow. The effects of viscosity on the regional differences in flow within the vessel have also been described[6,7]. In vitro, blood is normally 3-4 times as viscous as water. A Newtonian fluid, like plasma, demonstrates constant viscosity at all fluid flows. Blood, however, contains suspended cells that alter the fluidís behavior so as to make it non-newtonian. Unlike plasma, blood demonstrates decreased viscosity at increased flow rates. As red blood cells are, by far, the predominant suspended cell in blood, changes in red blood cell shape or iron composition will substantially alter blood viscosity[8]. As blood is a non-newtonian fluid, the parabolic flow pattern (i.e. faster in the center and slower along the walls) demonstrated by Newtonian fluids is not observed. This non-newtonian behavior of blood has physiologically beneficial effects. Blood is able to offer little resistance to flow in vessels with high flow velocity such as large conduit arteries, but is able to offer substantial resistance to flow in vessels with low flow velocity such as small arterioles. This slow movement of blood cells facilitates gas

exchange and interaction with endothelial cells. Interestingly, an effect related to small tube size occurs to prevent especially large viscosities from occurring at the very slow flow velocities characteristic of capillaries[9]. Poiseuilleís law can also be used to consider the effect of blood viscosity on the shear stress experienced by the vessel wall. At constant shear strain, shear stress increases with increasing viscosity. Thus, the sliding force exerted on the vessel wall increases with increasing viscosity of the fluid.

The heart

Whereas a consideration of the blood involves only its viscosity and non-newtonian nature, a consideration of the heart involves pressure and cardiac output. For the purposes of this study, the heart can be viewed as a pump that at a regular rate injects a specific amount of fluid at standard pressure into the vasculature. The pulsatile nature of the heart results in the pressure within the arteries alternating between a high pressure experienced during contraction of the left ventricle (systolic pressure), and a lower pressure experienced during the distention of the left ventricle (diastolic pressure). The pressure difference between the systolic pressure and the diastolic pressure is referred to as the pulse pressure. It is the action of the heart that creates the pressure gradient described in Poiseuilleís law. The left ventricle contracts with a force that is sufficient to overcome the resting pressure of the vasculature and the viscosity of the blood. The work done by this muscle contraction results in the movement of blood into the aorta and an increase in aortic pressure. The amount of blood forced from the left ventricle into the vasculature with each beat of the heart is called stroke volume. Stroke volume is dependent on the difference in volume of the left ventricle when distended (diastole) and when contracted (systole), though it is slightly less than this difference because of a small

reverse flow of blood back into the left ventricle before closure of the bicuspid valve. Cardiac output is the amount of blood pumped by the heart in one minute, and is dependent on both heart rate and stroke volume. (Cardiac output = heart rate x stroke volume), but the linear relationship is only true at physiologic heart rates. Stroke volume drops substantially with especially slow heart rates[10]. Similar to Ohmís law, the mean arterial pressure of the heart is determined solely by cardiac output and total vascular resistance (Mean arterial pressure =cardiac output x total vascular resistance). Though it has been suggested that pulse pressure may be a better indicator of cardiovascular disease[11], mean arterial pressure remains the focus of most blood pressure management therapies.

The vasculature

Structure and function

The vasculature is a hierarchical system of distensible tubes. The levels of the vasculature are defined by the initial branching of large arteries into smaller arteries, then arterioles, then capillaries, then progressive merging into venules, then smaller and ultimately to large veins. The composition of each blood vessel is critical to its function in the hierarchy[12]. Blood vessels are composed of specifically arranged cell types in a matrix that contains collagen, elastin, and proteoglycans. The dry weight of an artery is approximately 30% of the total weight, with collagen and elastin typically being 50-75% of the total dry weight[13]. Proteoglycans, consistent with their function as space fillers, take up the majority of the extracellular space in arteries despite their low contribution to total weight. The proteoglycans present in arteries include: versican, biglycan, and

decorin. The glycosaminoglycans hyaluronic acid, chondroitin sulfate, heparan sulfate, and dermatan sulfate are also present[14]. The blood vessel wall has three distinct layers: the adventitia, the media, and the intima (Figure 1A, C). The adventitia is the outermost layer and is composed of loose connective tissue that is comprised mostly of collagens I and III[15]. The media is the fleshy middle layer that, in a healthy blood vessel, constitutes the bulk of the vessel wall. This layer appears as concentrically arranged layers of smooth muscle cells in a matrix that consists mostly of collagen type I, III, and V[15] separated by a fixed number of elastic lamina based on species and elastin expression[16]. The innermost layer is the intima. As the endothelium is only a single cell layer thick, the intima of a normal artery essentially does not contribute to the overall size of the artery, but does provide a smooth, non-thrombogenic surface for blood flow. The tight junctions that join the cells of a functional endothelium prevent plasma components from coming in contact with the thrombogenic medial layer. Between the medial layer and intimal layer is a basement membrane that contains collagen IV colocalizing with laminin, and collagen V[15,17]. Whereas collagen provides tensile strength, the elastin, which makes up the elastic lamina of the medial layer, provides the extensible material that allows blood vessels to demonstrate both circumferential and longitudinal stretch. As elastin fibers run along the length of the vessel, longitudinal stretch has been shown to be influenced by elastin content, circumferential stretch has been shown to be mediated by both elastin and collagen content[13,18]. An increase in collagen I content strengthens the vessel, but the ratio of collagen I to collagen III can also affect the vascular flexibility[17]. Conversely, the loss of type I collagen has been shown to substantially weaken arteries[19].

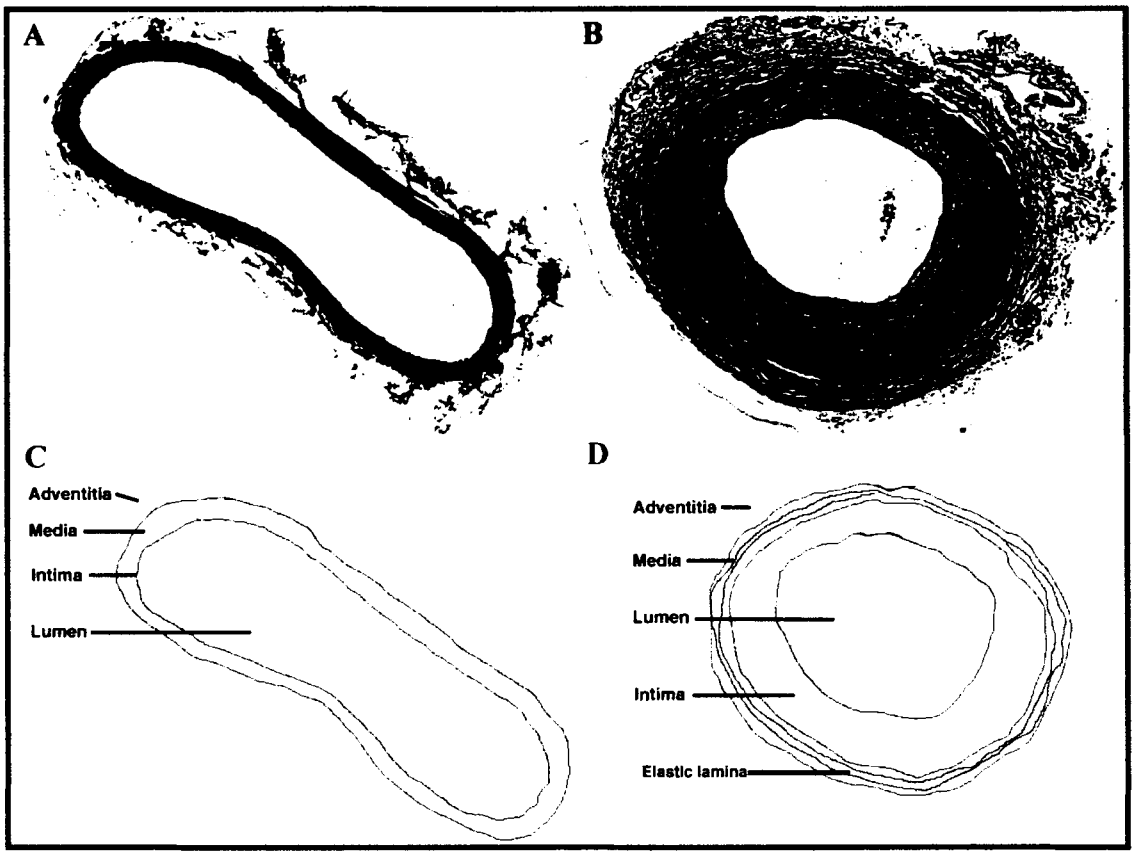


Figure 1. Neointimal lesion formation in the common carotid artery of rats following endothelial denudation. Cross-section of the common carotid artery from a rat following sham manipulation (A), and from a rat harvested two weeks after balloon catheter denudation (B). The sections were stained with Massonís trichrome stain indicating the presence of collagen in blue and proteoglycan in reddish purple. (C, D) Parts of the vessel. Balloon catheter denudation induces an injury response that is considered very similar to restenosis in humans. The elastic laminae appear as bands within the media.

The extensibility due to the elastin / collagen ratio[13] of the vascular wall allows the vessel to expand in response to an increase in pressure. This change in vessel size in response to pressure, termed vascular compliance, is critical to proper vascular function. The localized expansion of the aorta converts the kinetic energy of systole into a localized potential energy. The energy is then released over diastole as a smooth, passive constriction of the aorta that moves the blood forward, causing another localized expansion. The active expansion / passive constriction of the artery dampens the pulsatile flow of blood from the heart and is propagated along the vessel as a pressure wave. The velocity with which the wave propagates along the artery, called pressure wave velocity, is affected by both arterial compliance and blood viscosity. It should be noted that pulse wave velocity does not equate to the velocity of blood through the vessels, and this point allows distinction of the two functions of arteries: 1) to provide a conduit for blood flow, 2) to conduct the energy of the contraction of the heart necessary for movement of the blood. The specialization of the arterial network can be appreciated with the realization that different circuits of the circulatory system have different nutritive needs as well as different energy requirements. The circulatory system meets these different needs through the regulation of the lumen size and compliance of each individual artery. Whereas most distensible materials demonstrate constant elasticity in response to varying force (isotropy), blood vessels demonstrate different elasticities at different pressures (anisotropy). This anisotropy is considered to be the most important feature of blood vessels, as it provides the vessels with an efficient mechanism to regulate blood / energy transfer[20]. The aorta perhaps best demonstrates the specialization for transport of blood and energy. The aorta is tapered in size with the ascending aorta having the largest lumen on cross section, and the base of the abdominal aorta having the smallest. Numerous

arteries branch from the aorta along its length to provide blood to different circuits. Not only does the size of the aorta change along its length, but so does its compliance[20]. In this way, the aorta is able to provide both the correct amount of blood to each circuit and the correct amount of energy to properly move that blood through the given circuit. Interestingly, a smaller, reverse pressure wave is initiated at each transition at branch points. It is believed that these reverse pulse waves provide a feedback mechanism by which energy (heart rate and strength of contraction) is finely regulated throughout the circulatory system.

Vascular compliance also affects the amount of work the heart must do in order to circulate the blood. Cardiac output has been related to arterial compliance as follows[21].

Cardiac output = mean arterial pressure x (compliance / time constant)

As cardiac output increases linearly with an increase in compliance, increased compliance reduces the pressure at which the heart must operate to maintain cardiac output. As mean arterial pressure is related to total vascular resistance, the relationship suggests therapies that increase compliance should result in decreased resistance to blood flow. However, other factors, such as the alteration of total number of vessels must be considered when selecting therapy, and the relationship should be considered valid only in time frames that do not allow other compensations of the body. Vascular compliance has also been documented to affect pulse pressure[22-24]. Such an effect is intuitive with the realization that a rigid vasculature would not be able to dampen pressure.

The venous system has different specializations that reflect its function. Whereas arteries need to be able to handle the high pressures that result from being proximal to the heart, most of this energy is lost by the time the blood arrives in veins. Thus, the venous system transports blood at relatively low pressure with its primary function being return of the blood to the heart. Consistent with this lower pressure, veins demonstrate larger lumen areas and smaller medial areas than arteries at a comparable level. As the large driving pressure is largely absent, veins have specialized valves that keep the blood moving in one direction.

Regulation of vascular function

Several hormones are known to regulate vascular tone including vasopressin, endothelin, angiotensin, and nitric oxide. Vasopressin, also called anti-diuretic hormone, is a vasoconstrictor that is used at the level of the entire circulatory system to regulate blood pressure. Synthesized in the hypothalamus and secreted by the posterior hypothalamus, vasopressin in small concentrations stimulates water resorption in the nephron, but in larger concentration acts as a potent vasoconstrictor. Stimuli that lead to vasopressin secretion include: low blood pressure, high blood osmomolarity, and activation of the sympathetic nervous system.

The vascular response to the renin / angiotensin / aldosterone system is a method by which pressure is regulated at both the system level and at the level of the individual vessel. Renin is secreted in response to a drop in blood pressure or by a drop in plasma sodium concentration. The juxtaglomerular cells of the kidney, located on the afferent arterioles immediately adjacent to the entry to Bowmanís capsule of a nephron, sense the

drop in blood pressure,. The juxtaglomerular cells are believed to respond as stretch receptors to sense a change in blood volume. The juxtaglomerular cells are also positioned next to cells of the distal convoluted tubule. As these cells of the distal convoluted tubule are involved with the resorption of water / sensing of sodium, they are believed to communicate with the juxtaglomerular cells. In response to sensing low sodium or low blood pressure, the juxtaglomerular cells secrete renin into the blood. Renin acts as an enzyme on angiotensinogen, a protein secreted by the liver that maintains a steady blood concentration, to create angiotensin I. The conversion of angiotensinogen to angiotensin I occurs in the general blood supply throughout the body. Angiotensin I has mild vasoconstrictive properties that can provide a quick response to the low blood pressure that led to its formation. Angiotensin I is acted upon by angiotensin converting enzyme to form angiotensin II. Though angiotensin converting enzyme is found in the general circulation, its primary site of activity is in the lungs. It is estimated that in one pass through the lungs, 80% of the angiotensin I is converted to angiotensin II. Angiotensin II has two targets of action: 1) it acts on the general vasculature as a potent vasoconstrictor and substantially raises blood pressure quickly, 2) it acts on the zona glomerulosa of the adrenal gland to stimulate the secretion of aldosterone. Aldosterone acts on the nephron, specifically the distal convoluted tubule, to increase resorption of sodium ions, which also increases resorption of water. Aldosterone also has an action on the pituitary to increase secretion of vasopressin. Thus, vasopressin would target the distal convoluted tubule to increase sodium and water resorption as well. Angiotensin II stimulates collagen synthesis in smooth muscle cells in vitro[25]. Thus, angiotensin II regulates vascular compliance both in the short term, by smooth muscle tone, and long term, by altering the collagen / elastin ratio. In response to certain stimuli, angiotensin converting enzyme can be expressed by

cells of the vascular wall allowing individual vessels some control of vascular tone. Endothelin is also secreted by the endothelium where it acts as a potent vasoconstrictor providing additional control of individual vessels[26].

Another messenger molecule through which the endothelium regulates arterial function is nitric oxide. As nitric oxide exists as a gas at standard temperature and pressure, the detection of its presence is more difficult than for that of most other signaling molecules. Typically, the presence of nitric oxide is determined by change in the concentration of its precursors (L-arginine and citrulline). Nitric oxide in the vasculature is the product of three different enzymes: endothelial nitric oxide synthase, inducible nitric oxide synthase, and neural nitric oxide synthase[27]. As the mRNAs resulting from transcription of these genes are very similar, it is the promoter, and thus situations under which transcription occurs, that distinguishes the individuality of these genes. Endothelial nitric oxide synthase is an endothelium specific gene that is always expressed to some degree in endothelial cells.

Methods to measure vascular function

Laboratory methods exist to measure the movement of both blood and energy through the vasculature. In the case of transport of blood, relevant measurements must be made in vivo in a manner that does not interfere with blood flow. Doppler technology allows blood flow measurements to be made from a vessel with minimal disturbance. As sound waves are propagated through material interactions, blood flow through a vessel can be determined by measuring the shift of sound waves as they pass through a blood vessel.

Measurements of the movement of energy can be made with similar technology. Ultrasound with sufficient sampling rates and resolution can be used to measure pulse wave velocity. However, the determination of vascular compliance is not as straight forward. Though arterial compliance can also be measured using ultrasound[28] with systolic / diastolic changes in vessel diameter used to calculate compliance in the physiological pressure range, these methods rely on measurement of the change in vessel diameter occurring with each beat of the heart. Critique of the method centers on the lack of sufficient time for the vessel diameter to equilibrate at each pressure. Vascular compliance measurements are often measured in vitro using direct methods. Methods include the use of the wire myograph[29,30] and the pressure myograph[31,32]. Compliance is determined in a wire myograph by longitudinally passing two thin but rigid wires through the vessel lumen. A known force is then applied to the wires to separate them from each other, resulting in the circumferential stretch of the vessel. Measurements can then be made of the change in width of the vessel in response to the force to determine extensibility of the vessel. Arteries studied in a wire myograph only demonstrate stretch circumferentially as no change in the length of the vessel occurs. The pressure myograph takes a different approach. The arterial segment is ligated at one end, then sutured to a catheter at the other through which a known pressure can be applied. Like a balloon, vessels studied in a pressure myograph stretch both circumferentially and longitudinally. Comparison of compliance results of similar vessels in the different myographs reveals that deformation of the vessel alters compliance biaxially[33,34], and results obtained from one type of myograph may be very different from those obtained from the other. This

verifies that compliance measurements are geometry specific and has raised questions as to the validity of measurements from these devices[35]. Generally, the pressure myograph is considered to be more physiologically relevant than wire myograph[36].

Normal development of the vasculature

The vasculature forms early in development

The formation of the circulatory system begins following gastrulation, and involves complex interactions of extravasated plasma proteins and endothelial cell proteins induced by key cytokines[37]. Blood and blood vessels begin as hemangioblasts from the paraxial and splanchnopleural mesoderm forming concentrations in the gut, lung, aorta, and yolk sac. With the correct signals, these blood / vascular stem cells differentiate into endothelial cells and form primitive tubes. These blood islands then form the initial blood and capillary networks of the body. The expression of three families of vascular endothelium-specific growth factors are critical to the initiation of vasculogenesis: five members of the vascular endothelial growth factor family, four members of the angiopoietin family, and ephrin-B2. The interaction of these factors is complex, and the reader is referred to Yancopoulos et al.[38] for review of the actions of these factors. Additionally, Oettgen has reviewed the transcriptional regulation of blood vessel development (Figure 2). Vascular endothelial growth factor, originally called vascular permeability factor, appears to be the factor that initiates vessel formation, followed by Angiopoeitin-1 and ephrin-B2, which are involved in vessel maturation[39]. The expression of the ephrin-B4 receptor appears to be involved in the differentiation of arteries and veins[40].

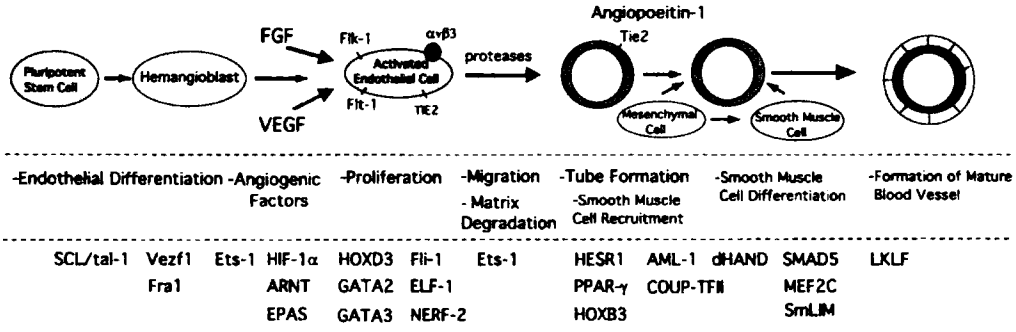


Figure 2. Growth factors and transcription factors involved in the development of blood vessels. The development of blood vessels involves the complex interaction of soluble messengers with differentially expressed receptors. (Figure taken from Oettgen[41].)

Vessel maturation involves both the recruitment of surrounding mesenchymal cells then their differentiation into vascular smooth muscle cells, and the remodeling of the extracellular matrix. The specific factors involved in maturation remains an area of investigation, though the continued expression of angiopoeitin-1 appears to be involved in the maintenance of vessel quiescence following vessel maturation[38]. The structural remodeling of the vessel occurs in response to the chronic physical forces to which it is exposed, such as pressure and shear stress, the specific function of the artery (transport vs. gas exchange, large vs. small artery), and compensatory mechanisms at the level of the individual artery and circulatory system. In vitro work suggests many factors may be involved. Growth factors have been shown to have specific effects on vascular cells and their supporting matrix. Consistent with the suggestion Schwartz and Liaw that fibroblast

growth factor- 2 overcomes endogenous inhibition of proliferation of endothelial cells then allows cells to enter mitosis in response to injury[42], fibroblast growth factor expression has been observed to increase cell number, but does not effect chondroitin sulfate content or dermatan sulfate content[14]. Similarly, epidermal growth factor acts as a mitogen but does not affect proteoglycan content. Opposing the action of these growth factors, insulin-like growth factor ñ1 and platelet derived growth factor do not act as an in vivo mitogen for smooth muscle cells, but to stimulate synthesis of chondroitin sulfate, dermatan sulfate and cell hypertrophy[14]. Interestingly, none of these growth factors affected heperan sulfate deposition[14]. Platelet derived growth factor, and transforming growth factor - β also appear to play a role in vessel maturation[41]. The expression of matrix metalloproteinases 2 and 9 occurs in the region around developing arteries, and null mutation results in early embryonic lethality presumably due to disturbed vessel formation[43].

The development of blood cells has been reviewed[44], with the specific lineage of different white blood cells being a topic of debate. Blood cell differentiation and development are also dependent on factors present in the area. Interestingly, the development of blood cells from hemangioblasts involves expression of several proteins that mark endothelial cell differentiation in response to many of the same factors. The differentiation of blood components with blood vessels is believed to involve either undiscovered differentiation factors, or local differences in concentration of known differentiation factors.

As compared to small arteries that form as small beds throughout the body during development, large arteries develop with the patterning of the early embryo. As described by Gilbert[45], following gastrulation, thickened areas of intermediate mesoderm fold to form lumens bilaterally. These cardiac primordia, though not containing blood, demonstrate pulsations. The tubes are drawn closer together as neurulation proceeds, and ultimately fuse to form the cardiac tube. In humans and mice, elongation of the central portion of the tube results in formation of an S-shape with left ñ right orientation. This S shaped structure develops into the 4 chambered heart. The anterior portion of the tube, linking with the aorta vessel network, forms the aortic arches surrounding the pharynx, while the posterior portion forms the vitelline veins. Meanwhile, the developing capillary networks expand to join the large central vasculature. In humans and mice, some of the aortic arches degenerate while others merge and expand to form the aorta, pulmonary artery, and carotid arteries. The capillary network of the chorion merges with the vitelline veins to supply nourished blood to the heart. Thus, the circulatory system forms from a merger of several circulatory subsystems, as opposed to the expansion of a central system.

Mature vessels remodel in response to physical forces

The metabolic needs of the body change throughout life as the body grows and exercise levels fluctuate. In order to meet these needs, the cardiovascular system must change as well. New blood vessels form in areas in areas of body growth, but instead of being replaced, established large vessels remodel their structure so as to meet the bodyís changing needs. Vascular response to acute needs is mediated by vascular tone in response to vasoconstrictors and vasodilators. Hormones, such as angiotensin II and vasopressin,

regulate the entire vasculature, while paracrine factors, such as nitric oxide and endothelin, provide local control of individual vessels. Vascular function in response remodeling to chronic needs is mediated by changes in vascular structure and smooth muscle cell reactivity. It is likely that the structural changes occur in response to expression of genes induced by certain vasoconstrictors[25,46].

The normal remodeling of the vasculature that occurs throughout life has been best demonstrated with the response of the vasculature to increased blood pressure. The increase in blood pressure disturbs the cardiac output- total vascular resistance balance[47], and is detrimental to the body as it places greater structural demands on the vasculature and shifts it out of the compliance range that is efficient for energy and blood transport. The remodeling of individual blood vessels in response to increased pressure depends on both the source of the pressure increase[48,49] and the position of the vessel in the vascular hierarchy[50]. Vascular remodeling may involve changes in gross vessel morphology, changes in the structural composition of the vessel, and local changes in the organization of the vasculature[51-53]. Changes in gross vessel morphology that have been observed in the presence of increased blood pressure include: increased medial area without changes in lumen area[54], increased medial area with decreased lumen area[55,56], and decreased lumen area without changes in medial area[31,57]. Both increased[56] and decreased compliance[31,58] have been observed in small vessels in response to increased blood pressure. In large vessels, both decreased compliance[59-63] and no change in compliance[50,64] have been observed in response to increased pressure. The collagen

content of vessels has been observed to increase with age and may partially explain the correlation of decreasing vascular compliance with increasing age[65-67]. In response to increases in blood pressure, the vascular content of fibronectin[68], and collagen V has been observed to increase[61], whereas elastin and proteoglycans[69] decline. The organization of the components also appears to be a source by which vessels can alter function as studies have shown both no change in the physical properties of vessel wall components[68,70] as well as physical changes[69,71,72]. The mechanism by which fibronectin participates in vascular remodeling was proposed to involve increased attachment of smooth muscle cells to the matrix[68]. In response to increased blood pressure, the vasculature has also been observed to reduce the number of small arteries and capillaries in a local area (rarefaction)[52,53].

Changes in smooth muscle cell reactivity have also been observed to accompany increased pressure[73,74,57]. The increase in pressure that accompanies aging was observed to correlate with decreased response to nitric oxide[56,67,75]. The source of hypertension appears to affect the response to specific vasoconstrictors / vasodilators is different in different animal models of hypertension[26,29,29,49,55].

Chronic obstructive vascular disease

Chronic obstructive vascular disease involves the remodeling of the vasculature in the presence of inflammation. The degree of inflammation differs between the specific diseases as well as the composition of the remodeled vascular areas. In general, a local

vascular lesion that includes both neointima formation and vascular remodeling (Figure 1B, D) characterizes the diseases. Whereas the intimal layer is normally thin and consists of a single layer of endothelial cells, the neointima may become so thick as to fill the lumen and consist of many layers of smooth muscle cell marker expressing cells. Whereas tight junctions form between the endothelial cells of the normal intima, neointima demonstrates substantial matrix deposition. The vascular remodeling that accompanies chronic obstructive vascular disease may be positive (increase in vessel total cross sectional area), or negative (decrease in vessel total cross sectional area) depending on the specific disease.

Examples of chronic obstructive vascular disease

Stenosis is perhaps the simplest of the chronic obstructive vascular pathologies and occurs in response to a chronic drop in blood flow (reduction in shear stress). The initial decrease in lumen area is due to vasoconstriction. Later, the reduction in lumen area involves permanent changes in the vessel wall. The stimulus that initiates inflammation is not known, but may be disruption of the tight junctions of endothelium from the long-term compression of the intimal layer allowing blood to contact the thrombogenic medial layer. Neointima formation varies from minimal to severe.

Unlike stenosis, restenosis involves neointima formation that is often accompanied by positive remodeling (an increase in the cross sectional area of the entire vessel)[76]. In this case, the lesion forms in response to the surgical excision of stenosed area or other disruption of the endothelium. Inflammation is induced by this direct injury to the vasculature. Restenosis occurs in 30-50% of all angioplasties[77].

Atherosclerosis has a complex etiology[57,78], and the reader is referred to Lusis for review[79]. In essence, this disease is a response to injury caused by oxidative modification of lipoprotein. Briefly, circulating lipoprotein is able to diffuse through the tight junctions of the endothelium and accumulates along the basement membrane. Sites of accumulation of lipoprotein are termed "fatty streaks" and are determined by characteristics of blood flow. In a process similar to that which will be described for all chronic obstructive vascular disease, macrophages (but not neutrophils) migrate to the subendothelial space to take up the lipoprotein. The lipoprotein along the basement membrane undergoes modifications including oxidation, lipolysis, and proteolysis so as to facilitate uptake by the macrophages. The modification of lipoprotein is believed to involve the enzymes myeloperoxidase, sphingomyelinase, and secretory phospholipase in the presence of several reactive oxygen species. The lipid-laden macrophages, now called "foam cells", alter the developing neointima so as to make it less stable. Lesion rupture generally occurs along lesion edges which are rich in foam cells and appears to involve an altered balance of matrix deposition / degradation in response to inflammatory factors. Interestingly, the incidence of myocardial infarction and stroke has been noted to increase during acute infection suggesting the involvement mediators of immunity with vascular disease.

Experimental models of chronic obstructive vascular disease

The remodeling of the vasculature that occurs in chronic obstructive vascular disease is similar to the remodeling of the vasculature that occurs throughout life in that it occurs in response to physical stimuli acting on the vessel. Whereas the vascular remodeling which occurs throughout life represents a system level adaptation to a

changing body, the remodeling associated with chronic obstructive vascular disease is a maladaptation to a local irritant. All of the chronic obstructive vascular diseases involve vascular remodeling in the presence of inflammation and most of the damage that occurs in lesion formation is due to the inflammatory response following the irritant. Though the presentation of the vascular lesion, underlying cause, and degree of inflammation vary in each chronic obstructive vascular disease, the vascular response to inflammation is believed to be similar in each case. Thus, any method that reliably induces an inflammatory response in the vasculature may be considered to be a model for the many aspects common to all chronic obstructive vascular diseases. Current models include animals which have been genetically modified to remove a vasoprotective protein (e.g. apolipoprotein E)[80], animals which have been genetically modified to over-express proteins which are harmful to vessels (e.g. renin)[81], infusion of chemicals which damage vessels (e.g. lipopolysaccharide)[82], and direct physical injury of the vessel. Direct injury methods have included cauterization[83], application of corrosive agents[84], electrocution[85], endothelial denudation[86], dehydration[87], cuffing[88,89], vibration[90], and ligation[91].

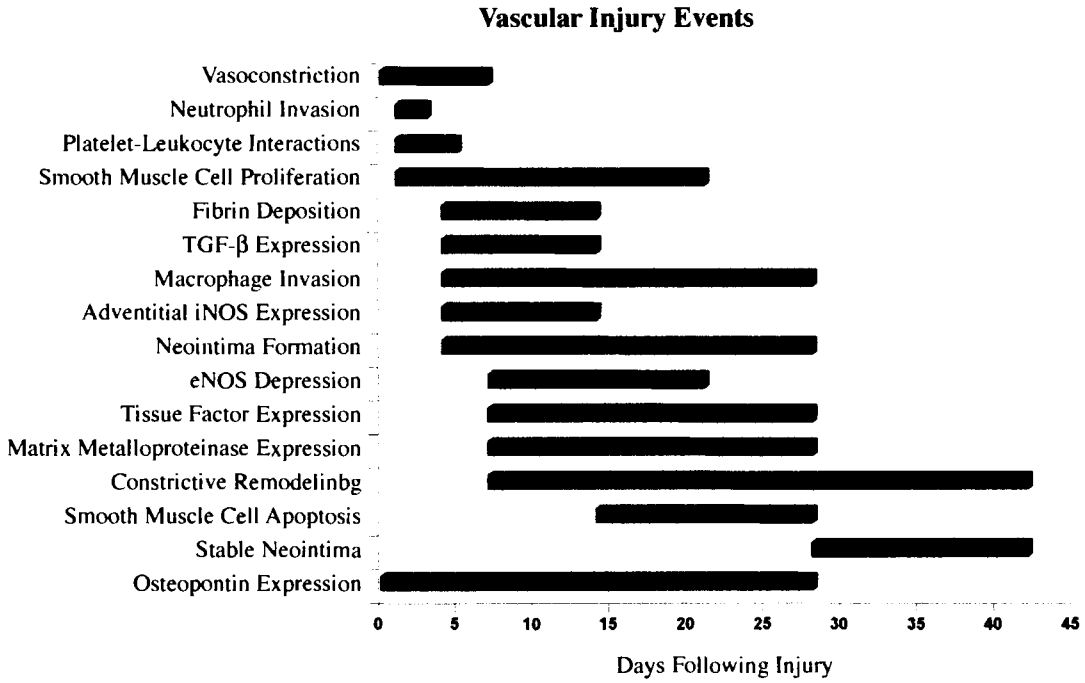


Figure 3. Timeline of events following vascular injury. Though there are many different vascular injury models, many induced events are believed to be common to all models. The precise timing of the events differs between models. INOS = inducible nitric oxide synthase, eNOS = endothelial nitric oxide synthase, TGF-β = transforming growth factor - β

Sequence of events leading to chronic obstructive vascular disease

Animal models that involve direct injury of the vasculature allow more precise temporal study of the injury response. Though specific timing of events varies between models, a general pattern of platelet / leukocyte aggregation followed by smooth muscle cell proliferation, migration, and extracellular matrix deposition can be identified. Studies that have observed the injury response at different times have been especially helpful in clarifying the processes of the injury response[92-94]. Using information gathered from studies using the many models, a general description of the vascular response to injury can be made by dividing the injury response into three periods: early, middle, and late (Figure 3).

The early period involves the first 4 days following injury. General responses to the procedure as well as responses specific to the injured vasculature characterize the immediate post-procedure period following induction of the inflammatory response. For example, matrix metalloproteinase -9 expression is observed in the common carotid artery of both ligated and sham manipulated mice immediately following surgery[95]. A second example is that arteries typically respond to any manipulation with a vasoconstriction whose duration seems determined by the level of trauma. Responses specific to the injured vasculature can be seen beginning the day after the procedure. The strong expression of adhesion molecules on the surface of endothelial cells is first observed soon after injury[93]. Platelets in conjunction with leukocytes can be seen aggregating near the endothelium[92]. The leukocytes involved in the initial interactions in the early period were determined to be neutrophils, that are quickly joined by macrophages[93]. The leukocytes extravasate and, in a reperfusion injury model, inhibition of neutrophil extravasation at

this time with annexin-1 has been shown to reduce neointima formation96. Proliferation of medial smooth muscle cells coincides with this leukocyte invasion[97], and smooth muscle cell migration from the medial layer to the intimal layer is observed to begin 2 to 4 days after the procedure[98]. Deposition of collagen type VIII[99], and fibrin deposition is seen along the vessel wall[92] also at this time, and, in models involving blood stasis, clot formation may occur between 2-3 days post procedure[92]. Along with an increase in transforming growth factor - β expression, tissue factor is observed to be expressed between the inner wall of the media and the forming fibrin layer[100]. By the end of this period, both neutrophils and macrophages are observed to have penetrated into the medial layer[93].

The period from 4 to 7 days following the procedure is marked by the appearance of a platelet / fibrin rich neointima extending into the lumen[92]. Interleukin-1 expression is observed in the media at this time, then peaks at day 10[101]. In models that demonstrate constrictive remodeling, the bulk of constrictive remodeling prior to day 7 is believed to be due to vasoconstriction94.

From 7 to 28 days, cell replacement is evident in both the neointima and media as both proliferation and apoptosis are observed. Smooth muscle cell proliferation is described as optimal for observation until day 14[92]. Smooth muscle cell apoptosis occurs after 7 days, and a decreased number of smooth muscle cells is observed at 14 days[91,94]. As this is the time that the observed constriction is increasingly unresponsive to vasodilator, Rudic et al. speculated the structural changes were due to smooth muscle cell

apoptosis[94]. Despite the loss in intimal cell number following day 14, intimal area has generally been observed to increase due to continued matrix deposition. As current lesion analyses do not consider the length of the involved arterial segment, the observed increase in intimal area may reflect a lesion that extends over a larger segment and not an actual increase in cross sectional area. At day 7, basal nitric oxide synthesis is observed to decrease at this time without a concomitant change in endothelial nitric oxide synthase levels[94]. Additionally, inducible nitric oxide synthase expression is observed in the adventitia from invading inflammatory cells[102], and expression of matrix metalloproteinase -2 is first observed to increase over its constitutive level at this time and remains elevated throughout this period[103]. Interestingly, the expression of this matrix metalloproteinase was shown to be related to flow restriction in the endothelial denudation model, with no change in expression in the case of high flow[103]. Angiotensin converting enzyme expression is observed to increase along the lumen border between 1 to 2 weeks following injury[104] and strong tissue factor expression persists in the neointima. By day 14, cells expressing smooth muscle cell markers are present throughout the neointima[92]. Transfoming growth factor-b returns to baseline expression at this time[97] and interleukin-1 is no longer observed after this time[101]. By day 28 post procedure, both neointimal formation and constrictive remodeling are believed to be stable in that little change in these important parameters occurs[91]. The application of vasodilator does not alter lumen area at this time, implying the vessel to be rigid.

Many players mediate the vascular injury response

The vascular lesions of chronic obstructive vascular disease form in response to the interactions of several cell types, secreted messengers, and modification of the extracellular matrix. Though it is believed that neointima formation results from smooth muscle proliferation in the media followed by migration to and further proliferation in the intima, the migration of these cells has not been proven. Smooth muscle cells in the intima display the smooth muscle cell markers of medial cells[91], but they also display a markedly different morphology. Whereas the nuclei of medial smooth muscle cells are large and oval shaped on vessel cross section, the nuclei of the intimal smooth muscle cells appear small and punctate in vessel cross section. Alternatively, it has been suggested that the intimal smooth muscle cells are the result of differentiated adventitial fibroblasts that migrated to the intima[105]. Although intimal cells appear in the absence of endothelium, it has also been suggested that the expression of smooth muscle cell markers represents a loss of differentiation of endothelial cells[105]. This question as to the origin of neointimal cells could be partially addressed with the use of mice containing an inducible smooth muscle specific reporter construct in which the reporter is expressed then inhibited just prior to injury. The appearance of reporter expressing cells in the neointima would indicate migration from the media.

Cells

Endothelial cells appear to play a multi-faceted role in the response to injury. Endothelial cell expression of cell adhesion molecules allows homing of inflammatory cells to the site of injury. Cell adhesion molecules belonging to the integrin, selectin, and

immunoglobulin superfamily are expressed following injury, and loss of function of Mac-1[87,106,107], selectins (E,L,P)[108,109], intracellular adhesion molecule-1[110,111] results in decreased neointima formation. Platelet binding to sites of vascular injury is initiated with the binding of platelet glycoprotein Ib-V-IX to von Willebrand factor on endothelial cells. P-selectin and Mac-1 are involved in the interaction of platelets with white blood cells[109,112]. Despite the presence of the endothelium, the compressive force associated with constrictive remodeling may directly interfere with the ability of the endothelium to prevent the thrombogenic media from coming in contact with blood. The interaction of platelets and leukocytes suggests the contact activation system may be involved in mediating the vascular response to injury. Support for this possibility is provided by the observation that overexpression of kallikrein results in diminished macrophage infiltration[113] and neointima formation[114,115]. Endothelial cells are a critical source of both vasoconstrictors (endothelin) and vasodilators in this system. Additionally, endothelial cells express different growth factors in culture than in vivo[116] suggesting the disruption of the cells with injury may induce a different phenotype.

The inflammatory cells believed to participate in the injury response have not been well characterized. Kumar and Lindner described the observation of macrophages in both the intima and media using BMA BM8, a cell surface marker of macrophages, immunostaining of cross sections[91]. Since that time, neutrophils have been observed to be the predominant inflammatory cell type in the first three days of response[93]. Little has been done in addition to these studies to characterize the inflammatory cells mediating the response to carotid artery ligation. Questions remaining to be answered involve the

identification of other leukocyte types in the injury response, and the determination of the activation state of the invading leukocytes. The role of prostaglandin has been studied in this model only through a drug studies in which aspirin was shown to have no effect on the response to ligation[27,102].

Vasodilators and vasoconstrictors

As with normal remodeling of the vasculature, vasodilators and growth factors affect vascular remodeling in the presence of inflammation. The amount of vasodilator present in the vessel during the early stages of the response to carotid artery ligation may substantially reduce constrictive remodeling. The initial vasoconstriction in response to injury may be due to reduced expression of endothelial nitric oxide synthase, as nitric oxide expression is noted to be diminished and vessels changed to low flow do not demonstrate altered response to angiotensin, aldosterone, prostanoids, or endothelin. The loss of endothelial nitric oxide synthase function is believed to involve post-translational modification as mRNA levels remain the same94. Interestingly, smooth muscle cells become hypersensitive to nitric oxide at this time, and studies using nitric oxide donors[117] or calcium channel blockers, which increase nitric oxide118, have shown decreased neointima formation via a MEK/ extracellular signal related kinase pathway[119,120]. However, arguing against this role for nitric oxide, endothelial denudation results in an acute increase in diameter and compliance of rat arteries[67]. In addition to this possibly important role as a vasodilator, nitric oxide has been shown to inhibit smooth muscle cell proliferation / migration, enhance endothelial cell migration and reorganization[94,121], and has anti-aggretory effects on platelets. Mice over-expressing

endothelial nitric oxide synthase showed less neointima, no change in total area, had decreased expression of endothelial adhesion molecules and subsequently fewer invading inflammatory cells[94,102]. The other source of nitric oxide in this system is from inflammatory cells. Various leukocytes express inducible nitric oxide synthase, and upon activation can secrete relatively large amounts of nitric oxide[102]. Besides being a potent vasodilator, nitric oxide is a reactive oxygen species involved in the destruction of pathogens[122,123].

Another important regulator of vascular tone that appears to have effects on the injury response is angiotensin II. Angiotensin II has been demonstrated to be involved in atherosclerosis, hypertension and restenosis[124]. Though not secreted by the cells at the site of injury, angiotensin concentration at the site of injury is elevated due to the expression of angiotensin converting enzyme at the injury site along the lumen border[125], and inhibiting angiotensin converting enzyme limits neointima formation[104]. Angiotensin infusion lead to iron deposition on renal tubules, heart and coronary arteries, but neointima formation was blocked with an iron chelator[126]. In vitro Angiotensin II and tumor necrosis factor - α induce smooth muscle cell proliferation via cyclooxygenase-2 activation of mitogen associated protein kinase/ extracellular signal related kinase[127], and inhibiting elastin synthesis in smooth muscle cells[46].

Growth factors

The expression of several growth factors has been noted from endothelial cells, smooth muscle cells, and inflammatory cells following injury[128]. fibroblast growth factor -1 and fibroblast growth factor -2 have both been observed to alter the injury response. Infusion of fibroblast growth factor -1 results in increased neointima formation[104], while fibroblast growth factor -2 infusion following denudation lead to a 4 fold increase in proliferation[129]. These effects may involve angiotensin as fibroblast growth factor has been observed to induce angiotensin converting enzyme expression[104]. Blockade of fibroblast growth factor -2 did not affect neointima size, but substantially reduced constrictive remodeling[130]. As heparin is able to bind many growth factors including fibroblast growth factor, it is not surprising then that heparin inhibits smooth muscle cell proliferation and reduces neointima formation in a manner that was free of coagulation effects[113,131]. Neither vascular endothelial growth factor nor one of its receptors FLK-1 was detected after injury, but high levels of another vascular endothelial growth factor receptor, FLT-1, were seen after injury[129]. Consistent with the shear stress response element GAGACC in its promoter, platelet derived growth factor is expressed following injury in an injury model blood stasis. Epithelial growth factor expression has been observed following injury from enothelial cells, smooth muscle cells, macrophages, and T cells. The promoter of transforming growth factor - β also contains the shear stress response element and expression of transforming growth factor - β following injury has been observed to increase with increasing flow[97]. In vitro, transforming growth factor - β inhibits vascular smooth muscle cell growth and migration and inhibits expression of

proteins involved in matrix degradation (collagenase, elastase, urokinase)[132,133]. Following endothelial denudation, transforming growth factor - β and its type II receptor expression localize to the wound edge, and blockade of transforming growth factor - β type II receptor profoundly inhibits constrictive remodeling[134]. The effect is probably due to transforming growth factor - β induction of collagen I and III expression. Other hormones that appear to have an effect on the injury response are estrogen[135], and 25 hydroxy cholesterol[136].

Cytokines

Regulators of immune function also have been demonstrated to affect the injury response. In general, expression of proinflammatory factors leads to greater neointima formation, and expression of anti-inflammatory factors leads to decreased neointima formation. Tumor necrosis factor - α has been observed in atherosclerotic plaques and following vascular injury[101]. Tumor necrosis factor - α precedes smooth muscle cell migration and leads to increased intracellular adhesion molecule-1 expression. Following carotid artery ligation, mice modified to overexpress tumor necrosis factor - α demonstrate increased neointima formation, whereas tumor necrosis factor - α null mice demonstrate greatly reduced neointima[101]. Similarly, interleukin - 1 is expressed following vascular injury and interleukin - 1 receptor 1 null mice demonstrated 7 fold less neointima than wild type[101]. Monocyte chemoattractant protein has been shown to be chemotactic for macrophages, and blocking CCR2, the receptor for monocyte chemoattractant protein-1, decreases neutrophil and macrophage invasion leading to less

neointima formation[115]. Interestingly, infection of the vasculature with monocyte chemoattractant protein-1 expressing virus does not lead to white blood cell accumulation at the walls of the vasculature[137] demonstrating the complex regulation of the vascular injury response. Prostacyclin expression reduces neointima formation due to its ability to inhibit smooth muscle cell proliferation and prevent platelet accumulation[138]. Some aspects of the injury response have been shown to be model specific as interleukin -10 expression has been shown to block atherosclerosis, but following injury interleukin -10 null mice did not demonstrate the expected increase in neointima formation as compared to wild type. Though dexamethasone inhibits macrophage accumulation after injury[139], primary cell lines derived from neointima showed decreased glucocorticoid receptor[140] suggesting that the steroid has less effect on cells of the neointima.

The extracellular matrix

Vascular injury also results in substantial changes to the extracellular matrix. Tai examined 68 genes involved in synthesis and breakdown of extra-cellular matrix using real time polymerase chain reaction and found 47 were differentially expressed following injury[141]. The matrix of the neointima is initiated with the deposition of fibrin along the lumen border. It is possible that the majority of early neointima is simply clot attached to the vessel wall. Consistent with this view, early neointima is observed to be eccentric, vary substantially in area, and have large portions of the neointimal area occupied by trapped red blood cells. Substantial remodeling in terms of both structure and composition involving fibrinolysis[84] results in later neointima appearing substantially

more dense and concentric. Fibrin has been shown to be a substrate for both endothelial cell and smooth muscle cell migration[92], and neointima formation was observed to be substantially decreased in a carotid artery ligation study using fibrin deficient mice[92]. Consistent with these results, inhibition of tissue factor by overexpression of tissue factor pathway inhibitor resulted in decreased neointima formation.[100]. On the other hand, overexpression of plasminogen did not alter neointima formation, but did alter the response of the media[89]. The collagen fibers that form in the neointima are thicker than those of the uninjured intima and have been shown to be thrombogenic[15]. Collagen type IV, present in the basement membrane underlying the endothelial cells, but not in uninjured intima, is present in neointima[15]. In contrast, mature atherosclerotic plaques contain mostly collagen I and collagen III, moderate amounts of collagen V, but no collagen IV[142]. However, collagen type IV may be found, along with collagens I, III, V in the fatty streaks that occur earlier in the development of plaques. Additionally, vascular injury induces a decrease in elastin[143], resulting in vessel stiffening[32].

Many components of the extracellular matrix that are expressed in response to injury contain the sequence arginine-glycine-aspartic acid. Examples include fibronectin, vitronectin, collagens, thrombospondin, fibrinogen, von Willebrandís factor, and osteopontin (OPN)[144]. The sequence is known to mediate interaction with integrin receptors, and, in vitro, changes to the sequence around arginine - glycine - aspartic acid affects binding to different receptors[144,145]. Evidence suggests that the $\alpha V\beta 3$ integrin is especially important in interacting with these extracellular matrix components. Studies

have shown this integrin to be involved with tumor progression146, cell survival147, cell migration[148,149], cell shape[150], cell proliferation[151], regulation of matrix metalloproteinase expression[152], angiogenesis[153], vasodilation[154], and vascular remodeling[155-158]. Intracellular signaling involved with αVβ3 includes: phosphatidyl inositol 3 kinase, Phospholipase A, and calcium. The vasodilation of aortic rings in response to arginine-glycine-aspartic acid peptides requires αVβ3 and is mediated by phosphotidyl inositol 3 kinase / Akt[159]. Interestingly, phosphotidyl inositol 3 kinase is known to physically associate with αVβ3[159]. With regard to vascular disease, increased αVβ3 expression has been observed in lesions from patients with restenosis, atherosclerosis, and transplant vascular disease[155,160].

The expression of matrix metalloproteinase has been observed following injury[103] and may be a mediator of the injury response. As these enzymes are known to remodel extracellular matrix and breakdown basement membrane, they may be involved in the remodeling of the neointima, and movement of cells from the media into the intima. The differential expression of matrix metalloproteinase correlates with smooth muscle cell migration[152] and, though the size of the neointima remains the same, smooth muscle cell migration is blocked with the matrix metalloproteinase inhibitor GM6001[161]. In addition to these critical roles in the matrix modification, matrix metalloproteinases have also been shown to modify the functional activity of various matricellular proteins through cleavage and subsequent exposure of cryptic sites within these molecules[162]. Thus, matrix metalloproteinases may affect the injury response directly through enzymatic action on

matrix components, and indirectly, through the modification of signals to other response-mediating cells. Metalloproteinases identified so far include matrix metalloproteinase -2 and matrix metalloproteinase -9[103] which our laboratory has also observed to cleave OPN.

The work presented in this text

Goal

The work presented in this text was funded by the American Heart Association (Grant #0250150N) with the broad goal of advancing our understanding of the processes that underlie chronic obstructive vascular disease such that interventions can be developed to reduce the associated morbidity and mortality. The specific goal of the project was to mechanistically describe the contribution of OPN to the vascular injury response. As will be described in chapter 3, OPN expression is known to be regulated by factors expressed in response to vascular injury, and high levels of OPN expression have been observed in chronic obstructive vascular disease. OPN is an arginine-glycine-aspartic acid containing protein that typically localizes to the matrix where it has been shown to alter the activity of attached cells. With regard to inflammation, OPN may be either pro-inflammatory or anti-inflammatory depending on the type and state of the tissue that produced it. Given, as described in previous sections, that vascular injury recapitulates many of the factors involved in chronic obstructive vascular disease, and the severity of the injury response generally correlates with degree of inflammation, we reasoned that the expression of OPN would have an effect on the vascular injury response. We further reasoned that determination of mechanism of its action would provide key insights into the pathological processes that underlie chronic obstructive vascular disease.

Hypothesis and strategy

We sought to test the following **hypothesis: The presence of OPN affects the formation of neointima and constrictive remodeling associated with vascular injury.** We suspected the loss of OPN would result in a diminished inflammatory response leading to reduced neointima formation. To test the hypothesis we chose the following **strategy: Compare the injury response induced by carotid artery ligation of wild type mice to mice with a null mutation of the gene encoding OPN at time points known to be critical to lesion development.** As described in previous sections, the response to vascular injury involves the interaction of many different factors at specific times. We believe that such complexity can only be accurately modeled in vivo, and determination of mechanism of action requires observations at different stages of lesion development to isolate the timing of OPN action. Additionally, as the vascular response to injury is complex, we suspected large variation in injury response within groups would require a large number of observations from subjects in a controlled environment. These experimental needs preclude the use of human subjects. Mice are commonly used as human models in the study of disease as, with respect to evolution, they are closely related to humans, can be obtained in large numbers, and their environment can be tightly controlled. The large vessels of mice are relevant for this study, as they are similar in size to human small arterioles, a possible site of disease. Mice have the added advantage of a ready availability of genetically modified lines. Indeed, as OPN null mice are available, and, as this is an exploratory study, subjects more closely related to humans are not required, we considered mice to be the ideal organism to use in this study. As compared to other vascular injury models, carotid artery ligation has

been shown to be a relatively simple means of reliably inducing a consistent vascular injury response in mice. The method has the additional benefit of being the most commonly used method in mice facilitating the incorporation of this work with that of others.

Overview

The work presented in this text makes a significant contribution not just to the study of chronic obstructive vascular disease, but to the general study of vascular biology as it identifies OPN as not only having a role in the response to vascular injury, but in the function of normal, healthy arteries as well. Additionally, the work demonstrates the first application of regression to the analysis of carotid artery ligation data that, due to increased comparative power and quality of description, should facilitate future experimentation and interpretation of studies.

Chapter 2

A REGRESSION MODEL OF THE RESPONSE TO MURINE CAROTID ARTERY LIGATION

Chapter 2 Introduction

Chronic obstructive vascular pathology (e.g. stenosis, restenosis, arteriosclerosis) remains a widespread health problem and the largest contributor to the morbidity and mortality of both men and women in developed nations[98]. These illnesses each involve a response to physical stimuli, such as oxidative injury or change in shear stress, and inflammation resulting in vascular remodeling and neointima formation. As this pathology has been shown to involve complex regulation of several cell types there continues to be great need for in vivo models of vascular pathology. As described in chapter 1, there are many different animal models of chronic obstructive vascular pathology including models that involve physical manipulation as well as animals that, due to genetic background, spontaneously demonstrate pathology. Each of these models has certain advantages and disadvantages that must be considered when selecting a model of vascular injury. Though the pathological processes may be similar, care must be taken in the interpretation of experimental data that may or may not reflect aspects common to all models. For example, OíBrien et al. observed the proliferation of smooth muscle cells in response to endothelial denudation is different than that observed in atherosclerotic plaques[163]. Additionally, as exemplified by Rogers et al., changes in experimental technique such as route of drug delivery may profoundly affect the vascular injury response[131].

Carotid artery ligation of mice

Murine models of vascular injury are especially useful in determining the contribution of specific gene products to the vascular injury response as they take advantage of the ready availability of genetically modified mice. Comparison of the

vascular lesions of wild type mice to those of modified mice is one method that allows the proteinís contribution to the injury response to be determined in a system free of neutralizing antibodies. The complete ligation of the carotid artery is one method of inducing an injury response in mice. Originally described by Kumar and Lindner[91], this model has the added advantage of being simple to perform. A small incision is made which allows exposure of the left common carotid artery. The artery is then ligated with suture just proximal to its bifurcation into the internal and external carotid arteries (Figure 4). Though the procedure does create a crushing injury, it is the loss of blood flow that is believed to induce the vascular injury response. Since its original description, this model has since been used to identify the role of plasma protein systems[84,114,115], cytokines[101,164] and physical forces such as pressure[118] and shear stress [91] in the vascular remodeling response.

Several variations of the carotid artery ligation model have been used in specific analyses. Song et al. performed ligation of the left common carotid artery in conjunction with endothelial denudation of the right common carotid artery in order to observe the effects of increased flow on the loss of endothelial cells[97]. Rudic et al. ligated the left external carotid artery in order to observe the response of left common carotid artery to reduced shear stress in the absence of injury[94].

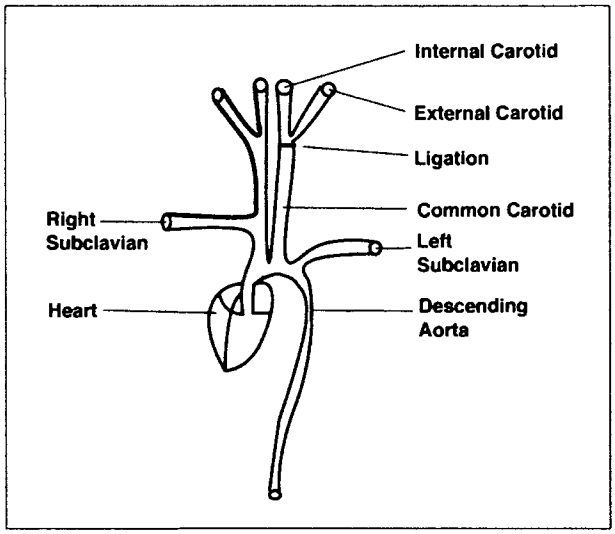


Figure 4. The site of the ligation in the carotid artery ligation model. The relative ease with which the mouse genome can be manipulated provides a powerful tool with which to study the role of various genes in the arterial response to vascular injury. However, the blood vessels of mice are very small and as such preclude the use of balloon catheter denudation to induce vascular injury. Carotid artery ligation is a means of inducing a vascular injury in mice similar to the response induced by balloon catheter denudation. In the carotid artery ligation procedure, the left common carotid is ligated just proximal to the branching of the internal and external carotid arteries. Because blood from the left and right common carotid arteries combine at the Circle of Willis, either can be completely occluded without causing significant harm to the animal.

Previous work has shown that the response to carotid artery ligation differs in mice of different backgrounds implying the response to carotid artery ligation involves the interaction of gene products from multiple loci[165]. Interestingly, neointima formation and constrictive remodeling were found to be separable responses. Some strains of mice demonstrated large neointimas with little constrictive remodeling, and vice versa[165]. Thus, the selection of genetic background in which to perform the study is critically important as some strains show a marked response while others show little response. The variable response of different strains suggests this model may have application in quantifying the contribution of different loci to the vascular injury response.

Problems in analysis of the vascular response to carotid artery ligation

Whereas the vascular lesions in response to other direct injuries, such as endothelial denudation, provide a relatively constant response over the involved arterial segment, murine carotid artery ligation induces a response which is generally greatest at the point of injury, then gradually diminishes over the involved arterial segment (3-5 mm from the site of ligation). Additionally, the blockage of blood flow following complete ligation of the carotid artery occasionally results in thrombus formation. If thrombus does occur, it typically occurs in the 1 mm segment closest to the ligature. To avoid the additional variable of thrombus formation, the 1 mm arterial segment closest to the ligature is typically discarded. The change in response with distance from the site of ligation and potential for thrombus complicate analysis of carotid artery ligation data. Analyses of the lesions induced by carotid artery ligation have been done in one of two ways: determination of mean response over the involved segment, or limiting analysis to a specific portion of the segment. Both of these methods of analysis have problems that

limit their interpretation. The representation of a predictably changing response with a mean does not indicate important aspects of the data and results in excessively large standard deviation. Limiting analyses to comparable regions begs the question as to how comparable regions were determined as inspection of data prior to analysis violates statistical premises. Both Yogo et al.[27] and Sindermann et al.[166] recognized the need to consider the effect of distance from the site of ligation in the analysis of response in this model. In this study we attempt to build on their work with the presentation of a regression analysis of data from a carotid artery ligation experiment. This method of analysis provides non-graphical description of the injury response over distance that is more precise than previous methods, as well as a way to compare the injury response that provides statistical significance. The method demonstrates that distance from the site of ligation is a significant predictor of the injury response. As regression analysis is a commonly used statistical method, actual calculations are not presented, and the reader is referred to SAS linear regression procedures[167] for description of the actual calculations performed.

Chapter 2 Methods

Murine carotid artery ligation

The Institutional Animal Care and Use Committee approved all protocols. Mice used in this study were on a 129 x Black Swiss hybrid background and 16-24wk old males were used for experiments. Ligation of the carotid arteries of mice was performed as previously described[91] on a test group of six mice and sham operations were performed on a control group of six mice. Briefly, mice were anesthetized with a solution of

ketamine (80 mg/kg, Fort Dodge Laboratories, Inc.) and xylazine (5mg/kg, Lloyd Laboratories) injected intraperitoneally. The left common carotid artery was exposed through a midline incision of the neck, and completely ligated just proximal to its bifurcation into the internal and external carotid arteries. The incision was then closed with surgical clips.

Two weeks following ligation, tissues were perfusion fixed with 4% paraformaldehyde in 0.1 M / L sodium phosphate buffer, pH 7.3. Vessels were harvested and embedded in paraffin such that initial sections from the block came from the internal / external carotid arteries. At least 800 consecutive serial sections were cut from both arteries of each mouse (7 μm thick). Sections including the ligature were easily identified as they sectioned very poorly due to the clearly visible suture material.

Morphometric analysis

As every section was saved and numbered, the distance from the site of ligation (relative start site in the case of sham manipulation) and between sections was precisely known. Sections of the internal / external carotid arteries were discarded. As analysis of previous work using this strain showed that substantial change in remodeling could be observed in sections 200 mm apart, we considered sections 280 μm apart to be independent observations. Ten sections from each mouse collected every 280 μm starting at the site of the ligatiojn, thus representing an arterial segment approximately 3 mm long, were stained with orcein to accentuate the border between the layers of the vessel, and morphometric analysis was performed. Digitized images of these vessels were analyzed using image analysis software (NIH Image 1.60). The lumen surface, the perimeter of the neointima, and the perimeter of the tunica media were traced, yielding circumference and

area of the lumen, internal elastic lamina, and external elastic lamina, respectively. The medial area was calculated by subtracting the area defined by the internal elastic lamina from the area defined by the external elastic lamina. Intimal area was calculated as the area between the lumen surface and internal elastic lamina. As the shape of the cross sections is often distorted due to sectioning and the neointima may be unevenly distributed, lumen area could not be measured directly. Assuming the external elastic lamina was circular, lumen area was calculated by subtracting medial area and intimal area from the total area calculated from the circumference of the external elastic lamina (Table 1).

Area	Formula
Medial Area	= external elastic lamina area – internal elastic lamina area
Intimal Area	= internal elastic lamina area – traced lumen area
Total Area	= (external elastic lamina circumference)2 / (4p)
Lumen Area	= Total Area – Medial Area – Intimal Area

Table 1. Calculations to determine vessel areas

Data analysis

Data was analyzed using a standard PC with SAS v8.0 as described in results.

Chapter 2 Results

Data collection

Mice (129xBlack Swiss) were separated into a test group (n=6) which underwent carotid artery ligation, and a control group (n=6) which underwent sham manipulation. We chose to harvest the carotid arteries at two weeks after the procedure as this is a

commonly used harvest time in carotid artery ligation experiments. Data were then gathered as described in methods. Interpretation of the resulting data was initiated with scatter plots of the different vessel areas versus distance for each mouse, and mean values for each distance were calculated (Figure 5).

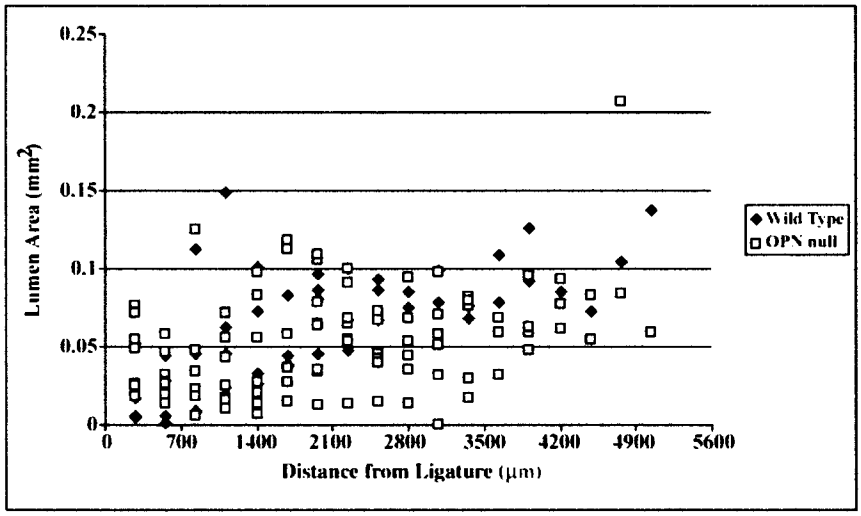


Figure 5. Example of scatter plot of morphometry measures. Measurements of lumen area are indicated as a function of their corresponding distance from the site of ligation. The data from both wild type and OPN null mice (discussed in chapter 3) appears well-behaved (continuous and differentiable).

Consistent with the description of Kumar and Lindner, the response to ligation was observed to be greatest near the ligature, then diminished with increasing distance[91]. As the scatter plots for both the individual mice and groups appeared well behaved (continuous and differentiable at all points), we considered the data suitable for regression analysis. The data was coded into SAS v8.0 including distance from the site of ligation for

each analyzed section. The contribution of variability with distance to the total variability was determined using Proc GLM as listed below. As changes in medial area, intimal area, lumen area, and total area all contribute to the injury response, separate models were calculated for each using the following command.

Proc GLM;

Model Total, Media, Intima, Lumen = ligature distance ligature*distance / solution;

The output listed both type I and type III sum of squares which showed distance from the ligature to be a significant predictor of lumen, intima, and medial area (Table 2). The output also provided parameter estimates for the linear regression model. With the exception of total area, these data indicated distance or the interaction of ligation with distance to significantly contribute to total variance.

Regression model of the vascular response to carotid artery ligation

We then sought to improve the models through fitting a curve to the mean response. Various functions were tested against the data for best fit. The scatter plots suggested y=arctan (x), y=e-x, and y=e- (x2) as possibilities for lumen and intimal area. Models were determined for total, medial, intimal, and lumen area using Proc Reg (Table 3) with the following command.

Proc Reg;

Model total media intima lumen = ligation distance ligdis;

Table 2. Much of the variation that occurs in the response to carotid artery ligation can be attributed to distance from the site of ligation. Analysis of variance reveals distance or interactions of distance with the presence of ligation to significantly predict the vascular remodeling response. The data shows a consideration of distance substantially increases the explained variance.

Total Area

Source	Degrees of Freedom	Type I Sum of Squares	Pr>F	Type III Sum of Squares	Pr>F
Ligation	1	0.09604	<0.0001	0.00585	0.2348
Distance	1	0.00009	0.8769	0.00376	0.3398
Ligation*Distance	1	0.00869	0.1486	0.00869	0.1486
Error	85	0.34774			
R-Square		0.23164			

Intimal Area

Source	Degrees of Freedom	Type I Sum of Squares	Pr>F	Type III Sum of Squares	Pr>F
Ligation	1	0.22187	0.0001	0.02780	<0.0001
Distance	1	0.02303	<0.0001	0.00621	0.0339
Ligation*Distance	1	0.00642	0.0311	0.00642	0.0311
Error	85	0.11364			
R-Square		0.31244			

Medial Area

Source	Degrees of Freedom	Type I Sum of Squares	Pr>F	Type III Sum of Squares	Pr>F
Ligation	1	0.01994	<0.0001	0.00198	0.0026
Distance	1	0.00009	0.5176	0.00067	0.0743
Ligation*Distance	1	0.00103	0.0283	0.00103	0.0283
Error	85	0.01755			
R-Square		0.54534			

Lumen Area

Source	Degrees of Freedom	Type I Sum of Squares	Pr>F	Type III Sum of Squares	Pr>F
Ligation	1	0.00039	0.6581	0.01815	0.0032
Distance	1	0.02285	0.0010	0.00189	0.3314
Ligation*Distance	1	0.01997	0.0021	0.01997	0.0021
Error	85	0.16799			
R-Square		0.20461			

With the exception of medial area, curvilinear regression models provided substantial improvement over the linear models. For example, the curvilinear of intimal area model had an r-square of 0.5548 using a x (1/7) curve as compared to 0.31244 for the linear model. The linear model proved best for medial area.

Model: Area = B_1 + B_2(Ligation) + B_3(Distance) + B_4(Ligated*Distance)

	Intima	**Total**	**Media**	**Lumen**
B_1	-0.0084	0.1236	0.0365	0.00889
B_2	0.2457	0.02074	0.01915	-0.08118
B_3	<0.0001	-0.000134	-0.00005	0.00009
B_4	-0.1074	<0.0001	0.00005	0.0012
N	1/7	3	1	3/4
R-Square	0.5548	0.4027	0.5453	0.4723

Table 3: Regression model of the response to carotid artery ligation. Curvilinear regression can be used to describe the response to carotid artery ligation with greater detail. All parameters demonstrated statistical significance.

Chapter 2 Discussion

As this study only examined the effects of ligation and distance from the ligation on the vascular injury response, only wild type 129 x Black Swiss mice were used. Within the model, the presence of ligation was described with a boolean variable (variable:

ligation), and the distance from the ligation was described with numeric variable (variable: distance). However, the analytical method can be easily extended to consider the effect of individual genes by comparison of genetically modified mice to wild type. For example, the comparison of wild type mice to mice lacking a particular gene can be performed with the inclusion of an additional boolean variable (describes the presence of the gene. See chapter 3 for analysis of the contribution of OPN to the remodeling response using this method of analysis.). Similarly the comparison of wild type to mice that over-express a particular gene product can be done with the inclusion of a numeric variable (describes the amount of gene product present). The method is also easily extended to consider interactions of test variables as demonstrated by the interaction of ligation with distance (variable: ligation*distance) in this study.

Our analysis demonstrates that variation with distance is a significant contributor to the total variance. Comparison of sum of squares shows distance contributes to 9%, 5%, 12%, and 66% of the explained variance for total, medial, intimal, and lumen area, respectively. The models also provide a quantitative description of the change in vessel areas with increasing data. Such information improves the description of the response as compared to previously used methods. In this case, the analytical method also expands on the description of two dimensional cross sectional areas by providing a consideration of the third dimension (distance from the site of ligation). The models could serve as the basis for the calculation of vessel layer volumes over an arterial segment.

As the vascular response is known to involve many factors, the flexibility of this method to consider variable interactions makes these methods especially suitable. In this study interaction is best demonstrated between the ligation procedure and distance in the model of neointima formation. No significant change in intimal area occurs over an arterial

segment in the absence of ligation. However, there is significant change in intimal area over an arterial segment in a ligated vessel. Phrased in terms of the intimal model, the dependence of a distance effect on ligation is expressed as interaction of ligation and distance being a significant predictor of intimal area. The clarification of the relationships of the many variables involved in the vascular response provided by this analysis should improve our understanding of obstructive vascular pathologies.

Clots alter the vascular response

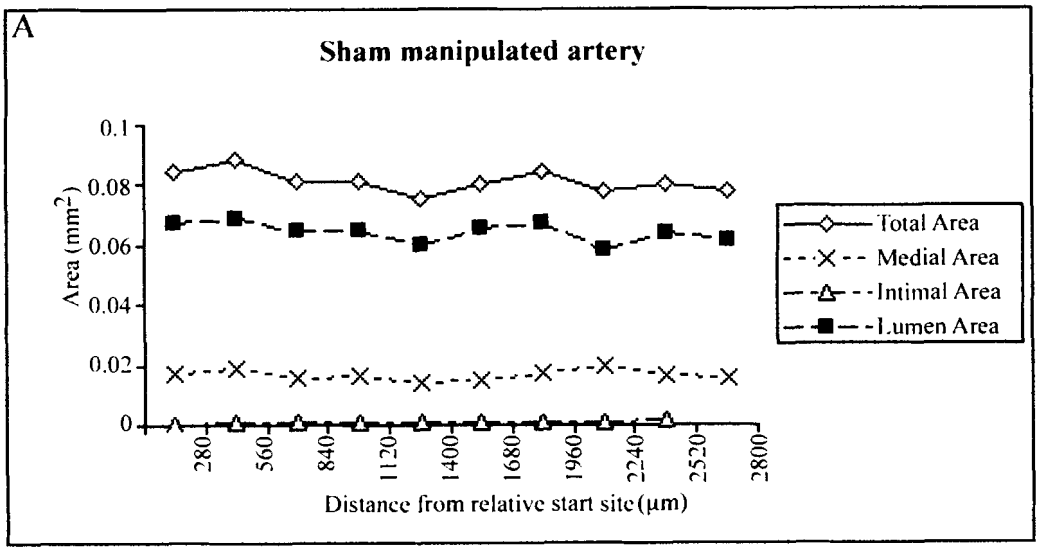
Further analysis of the data and regression models reveals that one mouse had a response to ligation that was much greater than those of the other ligated mice. Examination of the injury response in this mouse showed a large fibrous deposit in the lumen in the region less than 1 mm from the ligature. This deposit is probably the result of altered coagulation or a procedural problem and demonstrates the many influences involved in the vascular response. Interestingly, the outlying data came not just from sections containing the fibrous deposit, but also from sections observed to be free of the deposit. We interpret this as showing that the presence of the deposit alters not just the response of sections containing the clot, but the response along the entire vessel. Thus, the routine exclusion of the data from the one-millimeter arterial segment closest to the ligature removes the ability to detect an important source of response variation. To determine the effect of this outlying data on the analysis we repeated the analysis with the outlying data included. Though slight decreases in r-square values occurred for the linear models of the different vessel areas, inclusion of the outlier data did not alter the predictor significance of each variable. However, our attempt to improve the model with curve fitting was not as successful. The curvilinear model offered little improvement over

the linear model using the data set containing outlying data. Thus, the exclusion of data from mice that demonstrate an obviously atypical response to the ligation procedure is important to the development of good models of the vascular response.

Theoretical problems

As often occurs in the regression analysis of data from experiments, there are some theoretical problems in applying regression analysis to the data from carotid artery ligation experiments. Regression analysis requires observations to be independent. This condition is not met in these experiments as the response at one point in the artery is physically linked to the response at every other point. We addressed this problem with the selection of a distance between arterial points (280μm) over which previous work has shown substantial change can occur. Regression analysis also requires equal variance at each independent variable point. Examination of figure 6c shows that this condition is clearly not met. We addressed this problem with the use of weighted regression. However, as the method did not improve the model, we chose not to present this technique. We consider these theoretical problems to be minimal as compared to those of typically used methods (appropriateness of mean to describe changing data and inspection of data prior to analysis).

Figure 6. The vascular remodeling response varies with distance from the ligature. Medial, intimal, lumen, and total areas are shown as a function of distance from a relative start site for sham manipulated mice (A), and from the site of ligation for mice harvested 14 days following ligation of the carotid artery (B). While no significant change in the vessel occurs with distance in the sham manipulated mice, substantial variation in vessel areas occurs in ligated mice with increasing distance from the site of. If not accounted for, this variation with distance can complicate description and comparison of the injury response. (C) Standard deviations are shown for each mean intimal area calculated at different distances from the site of ligation. Variation in intimal area not only occurs with increasing distance from the site of ligation, but also occurs between mice of the same background. Note that the variation in intimal area at each distance is not the same. Similar results were obtained for medial, lumen, and total areas.


A
Sham manipulated artery
Area (mm2)
0.1
0.08
0.06
0.04
0.02
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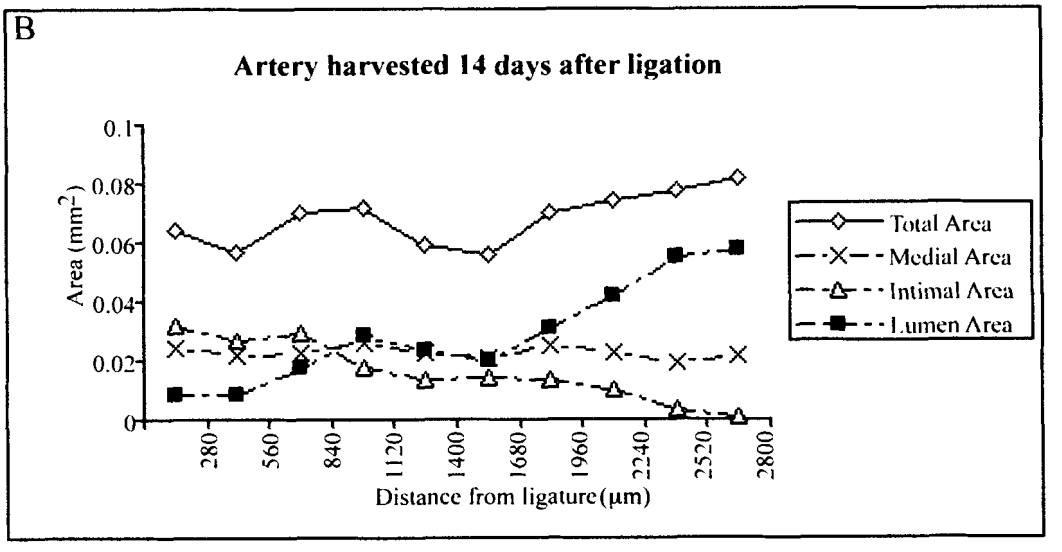
1680
1960
2240
2520
2800
Distance from relative start site(μm)
Total Area
Medial Area
Intimal Area
Lumen Area


B
Artery harvested 14 days after ligation
Area (mm2)
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0.08
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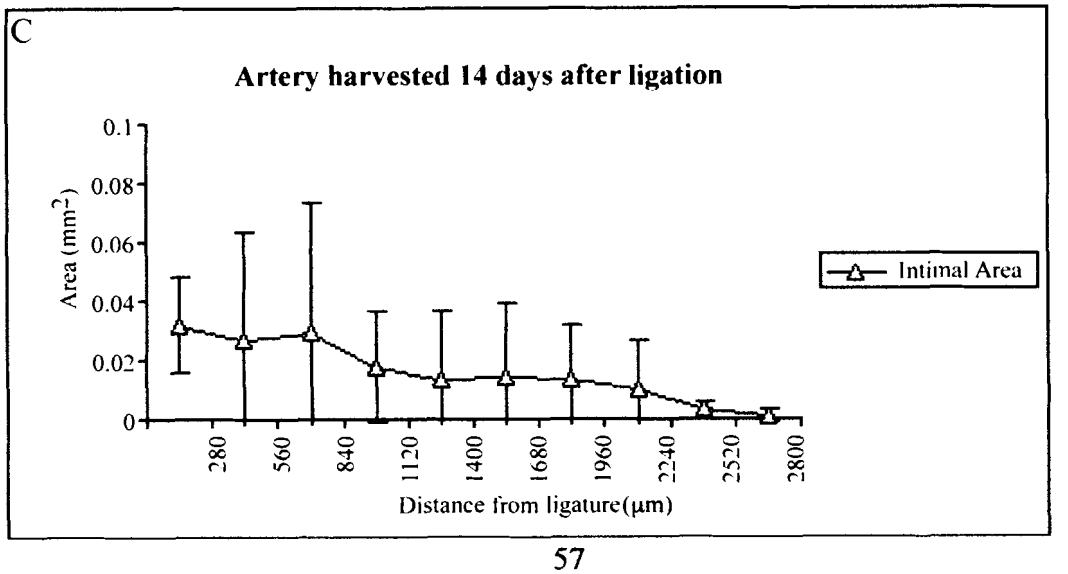
Distance from ligature(μm)
Total Area
Medial Area
Intimal Area
Lumen Area


C
Artery harvested 14 days after ligation
Area (mm2)
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0.02
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280
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1120
1400
1680
1960
2240
2520
2800
Distance from ligature(μm)
Intimal Area

Summary

The vascular response induced by carotid artery ligation is typically localized to the 3 to 5 mm region closest to the ligature. In this study the response was observed to involve approximately 3 mm. Several other models of obstructive vascular pathology involve a similar localized response. These pathology models might also benefit from regression analysis as the responses are multifactoral and may vary with distance along an arterial segment.

In summary, distance from the site of ligation was shown to be a significant predictor of the vascular injury response in carotid artery ligation experiments. By allowing simultaneous consideration of many factors, regression analysis provides both improved description of the response as well as increased comparative power through reduction of the unexplained variance.

Chapter 3

ALTERATIONS OF ARTERIAL PHYSIOLOGY IN OSTEOPONTIN NULL MICE

Chapter 3 Introduction

Both genetic and physiological factors have been shown to affect vascular physiology/pathophysiology. Platelets[92], leukocytes [87,92,106,109,168], endothelial cells[169,170], and smooth muscle cells[91,109,115,152] interact in a complex system regulated by plasma protein systems[84,114,115], cytokines[99,101,134], growth factors[130,134], and physical forces such as pressure[11,118] and shear stress [91,97,171] to remodel arteries in response to injury. In this study the arterial effects resulting from the removal of OPN are examined.

OPN, also known as early after T cell activation-1 (ETA-1), 2ar, secreted phosphoprotein1 (SPP1), and bone sialoprotein 1 (BSP-1), was originally isolated as a non-collagenous bone matrix protein[172] which was later found to be identical to a protein secreted by cancerous bone[173]. Early studies centered on a role for the protein in bone, but it was soon realized that OPN was transiently expressed in many different tissues undergoing remodeling following events such as infection, injury, or transformation.

The structure of SPP1, the gene that encodes osteopontin

The structures of the gene that codes for OPN, and the OPN protein itself have been reviewed[174-176]. OPN is the product of the secreted phosphoprotein (spp1) gene that exists as a single copy found on chromosome 5 near the ric genetic marker in mice, and on chromosome 4 at q13 in humans. Sequences homologous to human OPN have been found in rat, pig, cow, and chicken. The mammalian sequences share approximately 40% homology while the chicken sequence is only 19% homologous to human. The gene spans

approximately 7 kb, contains 7 exons, and a 0.5 ñ 2 kb promoter (Figure 7). Promoter elements include sites related to ras activation (The AP1 site is for the binding of a heterodimer of cFos / cJun), protein kinase C activation, a vitamin D response element, a Pu box, a GAGACC element shown to be responsive to shear stress in platelet derived growth factor95, and a CCAAT that binds a factor necessary for v-SRC stimulation of OPN expression[177]. Consistent with the presence of these elements, OPN expression has been shown to be induced by activated ras / MAPK178, calcium, and activated PKC[179]. Factors known to induce OPN expression include: parathyroid hormone, Vitamin D3, angiotensin II[180] (occurs via ERK/p38 pathway[181]), and transfoming growth factor - β[182], whereas estrogen, angiotensin converting enzyme inhibitors, angiotensin II receptor antagonists have been shown to decrease OPN expression[183]. Vitamin D induced OPN expression is suppressed by HES-1 in osteoblasts[184].

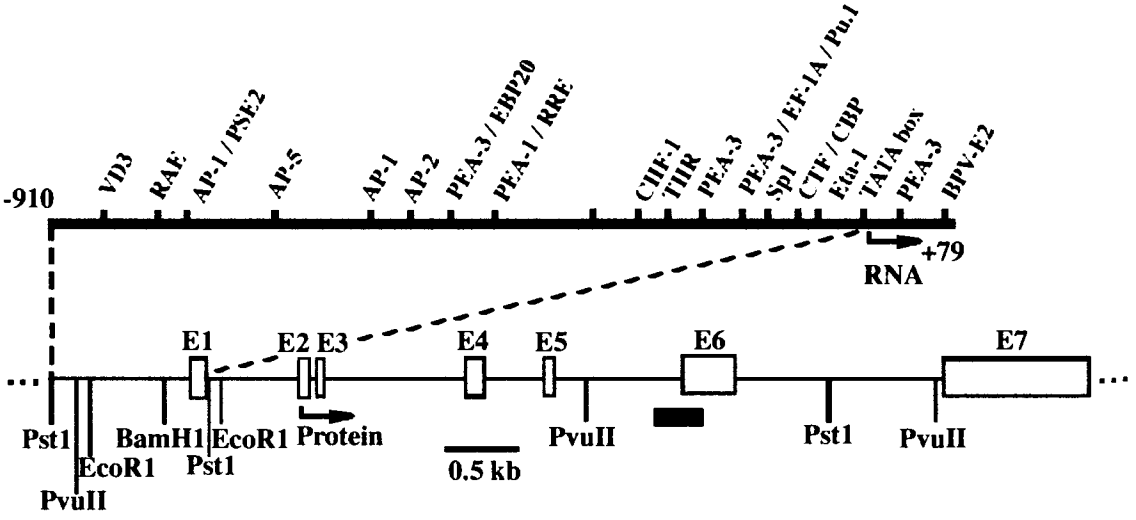


Figure 7. The structure of the murine spp1 gene. (Taken from Denhardt and Guo[175])

The structure of osteopontin protein

The OPN protein has an amino acid backbone that is 264 (chicken)[185] to 317 (rat)[173] amino acids in length depending on the species (parenthetical references to specific amino acids refer to the human protein). The protein is rich in aspartic acid, glutamic acid and serine residues making it quite acidic. The secondary structure has been predicted to contain 8 sections of alpha helix and 6 short beta sheet sequences[175]. Circular dichroism experiments suggest a random tertiary structure in which regions linking secondary structures are free to move[186]. Consistent with a flexible structure, no crystal structure has been reported. The protein contains a relatively large number of domains with potential biological activity (Figure 8). A transglutamination site is found in the N terminal of the protein that can interact with the enzyme to crosslink OPN to other proteins via glutamine ñ lysine covalent bonds. Calcium binding loops are found in the middle of both the N (aa 86-95) and C (aa 216-228) halves, and two heparin binding loops are found the C terminal half of the protein (aa 168-174, aa 298-305). The integrin binding arginine-glycine-aspartic acid sequence is found in the middle of the sequence (aa 158-162) close to an RS thrombin cleavage site[187,188] (aa168-169). Interestingly, a second integrin binding site containing the sequence SVVYGLR[189] (aa 162-168) is found between the arginine-glycine-aspartic acid sequence and the thrombin cleavage site. This second integrin binding site is cryptic for $\alpha 9\beta 1$ and a4b1 as it only becomes active upon cleavage of OPN by thrombin[190]. Additionally a ELVTDFPTDLPAT site (aa 131-143) upstream of the arginine - glycine - aspartic acid sequence can act as a second binding site for $\alpha 4\beta 1$[172].

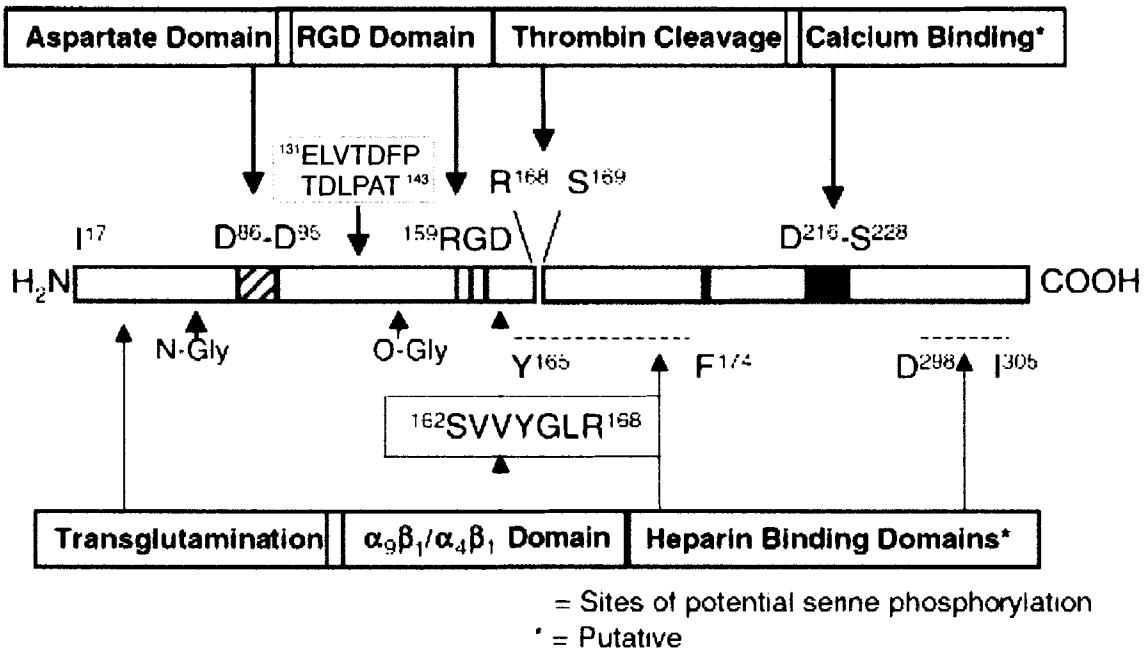


Figure 8. The structure of osteopontin protein. (Modified from Denhardt[197])

Modifications of osteopontin

Mechanisms that modify the structure of osteopontin include differential splicing, post-translational modification, cleavage, and cross-linking (Figure 9). Three OPN splice variants have been described, all of which involve the selective splicing of exon 3[191,192]. Each of the OPN splice variants contains an export sequence, and upon translation OPN enters the secretory pathway where it may be extensively modified. The post translational modifications are both tissue and cell state specific[193,194]. Modifications may include O-linked glycosylation (6 sites), N-linked glycosylation (1 site), phosphorylation (15 sites) and sialation (10 sites)[175,186,195]. The specific phosphorylation of OPN can be done in a cell free system[175,196] as Butler[186] described

that the ser -X- glu (12 sites) sequence marks a potential site for casein kinase phosphorylation of OPN, and the ser -X- glu sequence (2 sites) marks sites for phosphorylation by casein kinase II.

Specific post-translational modifications and interactions with other molecules that modify its conformation largely determine the biological activity of OPN. These observations imply there is a strict structure ñ function relationship in the action of OPN and support the belief that the different forms of OPN have different biological activity. Jono et al. demonstrated a phosphorylation requirement for OPN to mediate calcification of smooth muscle cells[198]. The binding of OPN by factor H alters the OPN structure so as to hide the arginine-glycine-aspartic acid sequence resulting in reduced inflammation[199]. Though OPN binding of calcium is not phosphorylation dependent[192], the binding of calcium also alters OPN conformation[200]. The ability of OPN to bind wells coated with collagen types I, II, III, IV, and V is calcium dependent[186,201]. OPN has been shown to bind fibronectin [202]. and osteocalcin and the binding of osteocalcin inhibits transglutaminase action on OPN[203].

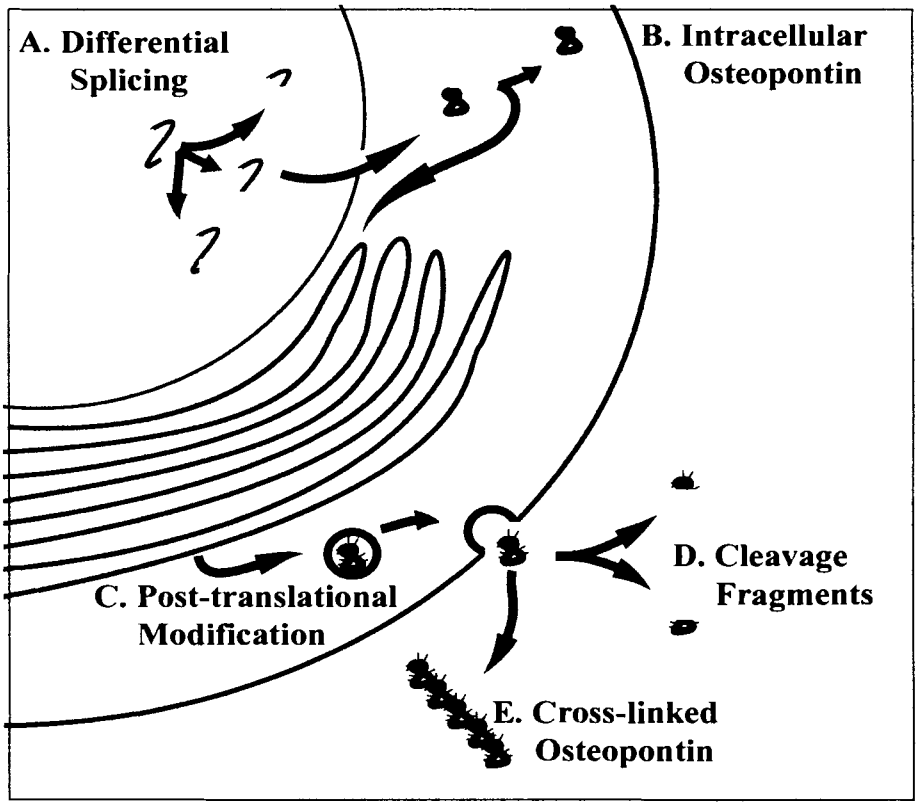


Figure 9. Osteopontin exists in many different forms. A) There are three known OPN splice variants. B) Though intracellular OPN has been described, C) OPN generally enters the secretory pathway where it may undergo extensive post-translational modification. D) Upon leaving the cell, OPN may be cleaved by matrix metalloproteinases or thrombin, or E) cross-linked by transglutaminase.

OPN is also structurally modified by cross-linking and cleavage of the protein. OPN is a substrate for transglutaminase (factor 13a)[195,204], which creates OPN multimers and links OPN to fibronectin[205]. The formation of OPN multimers results in increased collagen binding[201]. The cleavage of OPN by thrombin exposes the cryptic integrin binding site and yields fragments with different biological activity than that of full length OPN[188,206 207 208]. Other work in our laboratory has identified cleavage sites for matrix metalloproteinases 3 and 7 in the OPN sequence[209]. Interestingly, one of these sites is just four amino acids downstream of the arginine-glycine-aspartic acid integrin-binding site (aa 166), and is internal to the cryptic integrin-binding site. The cleavage effects of these matrix metalloproteinases on the cryptic integrin-binding site have not been determined. Additionally, our laboratory has also observed OPN cleavage by matrix metalloproteinase- 2 and matrix metalloproteinase - 9, but these cleavages have not yet been characterized. The cleavage fragments do not equate to splice variants.

Though OPN is typically considered to be a secreted protein, Zohar et al. used confocal microscopy to localize intracellular OPN outside of the secretory pathway[210], and has proposed this intracellular OPN to act as a survival factor[211]. Though intriguing, the published experiments are not convincing as they lack important negative controls.

Receptors for osteopontin

There are eight known receptors for OPN[212], seven of which are integrins (Table 4). Binding affinities have only been determined for the αV containing integrins, all of which occur in the nano-molar range[174]. As with most integrin mediated signaling,

intracellular signaling as a result of OPN binding is complex. Rather than signaling through the direct formation of a second messenger, integrins are believed to transduce signals through the formation of an intracellular complex involving the cytoskeleton whose specific three dimensional conformation results in enzymatic activity[213]. Key players in integrin signaling include: focal adhesion kinase, integrin linked kinase, caveolin, paxillin, and talin[214]. The ability of integrins to bind ligand is often believed to involve reversible modification of the intracellular domains of the integrin subunits (activation). Thus, integrins can exist in different states, and the ability of integrin to bind ligand is state dependent. The binding of OPN to platelets and lymphocytes requires this activation of the integrin[215, 216]. Pathways that have been shown to be activated in response to integrin signaling include alterations in calcium levels including both increases and decreases[175], alteration of pH, transient increase in protein phosphorylation, activation of protein kinase C, mitogen associated protein kinase and phosphatidyl inositol 3 kinase[215]. The biological effects resulting from interaction with OPN are mediated by distinct integrins[149]. Particularly notable is the aVb3 integrin requirement for motility[149] whereas other integrins are primarily involved with adhesion[149, 217]. The non-integrin receptor for CD44 has been shown to exist as many different splice variants, only one (exons v4, 5,6,9) of which serves as an OPN receptor[218-220].CD44 binding of OPN is involved in cell survival[221] and is required for OPN mediated migration[222-224].

OPN Receptor	Features
$\alpha V\beta 1$	Adhesive receptor[149]
$\alpha V\beta 3$	Involved in angiogenesis[297] and cell motility[215,149]
$\alpha V\beta 5$	Involved in cell motility[148]
$\alpha 4\beta 1$	Involved in leukocyte adhesion[298]
$\alpha 5\beta 1$	May be involved in the progression of atherosclerosis[299]
$\alpha 8\beta 1$	May be involved in matrix deposition/ adhesion[300]
$\alpha 9\beta 1$	Binds cryptic site exposed by thrombin cleavage[188]
CD44	Associated with tumor progression[220]

Table 4. Osteopontin Receptors

The biological role of osteopontin

OPN expression has been observed in bone, kidney, tumors, placenta, the cochlea, milk, and other bodily fluids[225-227]. The in vivo role of OPN has mostly been studied in mice. Both OPN transgenic[228,229] and knockout mice[230,231] have been described. In both cases, the mice are viable, fertile, with a "normal" phenotype. The normal appearance of these mice is not surprising given the transient nature of OPN expression in response to tissue remodeling. Also, there is much redundancy in the domains of matricellular proteins implying that the function of OPN, in most cases, can be fully achieved by a combination of other matricellular proteins[140]. The findings of studies that have observed alterations in response to the presence or absence of OPN are often contradictory, probably owing to the functional effects of OPN conformation. These contradictions and the diversity of the areas in which OPN is expressed make it difficult

to describe the function of OPN outside of the system being studied. Though the functions of OPN in specific areas of the body can be described, a general description of the role of OPN in the body remains conjectural[232].

Bone

In bone, OPN has been shown to both facilitate and oppose bone resorption. OPN has been shown to facilitate accumulation of osteoclasts at sites of bone resorption[233] and OPN null mice show altered osteoclast formation[231]. The expression of OPN is required for bone resorption in response to parathyroid hormone[234] and decreased mechanical stress[235], and OPN null mice are resistant to ovariectomy induced bone resorption[236]. Additionally, osteolytic tumor cells that are osteolytic upregulate OPN secretion in osteoblasts[237]. However, the effect of OPN on mineralization appears to be conformation dependent as treatment of OPN with phosphatase increases mineralization[175].

The induction of OPN in response to stress has been observed in bone cells. In vitro, OPN is induced with mechanical stimulation of osteoblast cells, and OPN null bone cells are defective in producing nitric oxide in response to pulsatile fluid flow[238,239].

Kidney

As the kidney is a vital organ involved in the maintenance of blood pressure and blood composition, the primary effects of OPN expression in this organ may cause secondary effects throughout the body. OPN is expressed in the developing tubule

epithelium[240] and is constitutively present in the loop of Henle and the distal convoluted tubule of adults[183,240]. OPN is differentially expressed by podocytes of the glomerulus in response to stretch[241], thus, as hypertension correlates with atherosclerosis[242,243], it is not surprising that increased OPN expression has been observed in the kidneys of atherosclerotic patients[244]. Consistent with these pressure effects on OPN expression, infusion of angiotensin II leads to increased OPN expression throughout the kidney[245].

OPN is also expressed throughout the kidney in tubulo-interstitial disease where its effects are harmful as OPN null mice demonstrate less injury[246] in response to induced disease. Conversely, OPN null mice showed reduced tolerance to ischemia in kidneys[247]. As described in chapter 1, the glomerular cells, juxtaglomerular cells, and cells of the distal convoluted are believed to communicate such that pressure sensed at the glomerulus can alter fluid resorption at the tubule. Expression of OPN in the distal tubule suggests a role the maintenance of blood pressure and possible mediation of flow through the glomerulus by inhibition of nitric oxide[248].

Inflammation

The expression of OPN has been shown to have both proinflammatory and anti-inflammatory effects[183,249]. In vitro, OPN has been shown to be expressed by and chemotactic for macrophages[250-252], and T cells[253]. Indeed, OPN may be the most abundantly secreted protein from T-lymphocytes[254]. Dendritic cells and Langerhan cells

also demonstrate migration toward OPN via a CD44/αV mechanism[255]. Consistent with OPN mediation of inflammatory cell migration, OPN null mice demonstrate a decreased number of macrophages in tubulo-interstitial disease[256], decreased ability to clear respiratory infections[257], and altered granuloma formation in response to sterile irritant104. Additionally, OPN has been shown to alter the interleukin -10 to interleukin -12 ratio[258] so as to promote inflammation, and T cells from OPN null animals have been shown to make less interleukin -10 and interferon gamma[259]. OPN expression induces B cell proliferation and expression of immunoglobulin[254]. However, OPN also demonstrates anti-inflammatory actions as it inhibits the synthesis of nitric oxide, a reactive oxygen species, from inducible nitric oxide synthase[122,260,261].

Cancer

The role of OPN in tumor progression has been studied also with seemingly contradictory results probably owing to the results of modifications or cleavage on protein function[162,206,262]. OPN expression has been observed in various tumors[263] and OPN expression has been shown to increase in response to transformation[264,265] leading to the use of OPN as a cancer marker[266,267]. OPN can be detected in the blood when expressed in high amounts, and high plasma levels of OPN have been shown to positively correlate with the metastatic potential of tumors[268]. Anti-sense blockade of OPN leads to decreased malignancy[269] and tumorigenicity[270,271]. However, the tumor inhibiting functions of OPN have also been demonstrated as OPN null mice show

accelerated tumor growth / progression[262]. OPN blocks 12-O tetradecanoyl phorbol 13 acetate induced anchorage independence and reduces tumorigenicity and metastasis[262]. Additionally, as described above, OPN has proinflammatory properties that should limit tumor progression.

Similar to this role in promoting metastasis, OPN is believed to play a role in embryonic implantation. OPN is expressed in decidua basalis where it aids the avoidance of immune detection of fetal cells[272]. As in cancer, the conformation of OPN is important as different charge forms affect action[273].

Vasculature

OPN is present in small amounts in uninjured arteries[274], but is abundantly expressed by smooth muscle cells, endothelial cells[141,274], and activated inflammatory cells[249,252,254] in injured arteries. Abundant OPN expression has also been observed in human atherosclerotic lesions[217] and thoracic aneurysms[275] aortic valvular lesions[276], left ventricular hypertrophy[277], and the presence of OPN in the blood following angioplasty was shown to positively correlate with detrimental post procedural events[77]. In vivo studies confirm the relevance of OPN during vascular remodeling and repair. Chiba et al. observed that hematopoetic over-expression of OPN resulted in increased atherosclerotic lesion formation[229]. The role of OPN as a physiological inhibitor of vascular calcification was demonstrated in studies showing that spontaneous vascular

calcification in the matrix of Gla protein null mice was significantly increased on an OPN null background[278]. Recently, the constitutive overexpression of OPN in mice was observed to result in increased neointima formation following cuffing of the femoral artery[279]. This overexpression of OPN in uninjured mice did not result in accumulation of leukocytes in the vessel wall, indicating that control of vascular inflammation is complex.

Though matrix metalloproteinase -2[95,103] and matrix metalloproteinase -9[103] are both expressed after vascular injury, and our laboratory has observed these matrix metalloproteinases to cleave OPN, western blot shows the small amount of osteopontin present in uninjured vessels exists as three widely separated bands, whereas following injury only the heaviest band remains[296].

Similar work

The in vivo work that most closely relates to the present study demonstrated anti-OPN antibody to inhibit neointima formation following endothelial denudation[280]. In this model, a catheter is inserted into an artery. The catheter is then inflated and pulled back such that the endothelial lining of the blood vessel is stripped away. In this model, OPN expression was initially observed two days following the procedure at the site of injury. Strong OPN staining was observed in the newly formed neointima at day 7 peaked at day 10, then slowly faded until absent by day 42.

This present study utilized a genetic OPN null murine model to test the hypothesis that OPN regulates vascular remodeling in vivo. Our studies show the first demonstration that endogenous OPN contributes to normal blood vessel structure and

function, and that OPN is a regulator of both constrictive remodeling and neointimal lesion formation. We provide evidence to support a model where OPN is mediating these effects through regulation of vascular compliance and the inflammatory response.

Chapter 3 Methods

Animals

The Institutional Animal Care and Use Committee approved all animal protocols. The OPN null mutant allele has been described281, and mice used in this study were 16-24 week old males on a 129 x Black Swiss hybrid background. Heterozygous breeding pairs were used to generate wild type or OPN null groups.

Blood count, hemodynamics, and vascular compliance

25ml of blood was obtained by retro-orbital collection (wild type: n=10, OPN null: n=10), diluted to 200ml, and then analyzed by flow cytometry (Cell-Dyn 4000). Blood pressure measurements were made using a Visitech BP200 tail cuff (wild type: n=29, OPN null: n=17). Following a one-week conditioning period, 30 measurements were made in a single daily session, over 5 days. The initial 10 measurements were discarded, and the last 20 averaged to give a single daily blood pressure measurement for each mouse.

Blood flow was measured using a Transonic flow probe with T160 computer interface (wild type: n=18, OPN null: n=17). Animals were anesthetized with Avertin (2ml of 2.5% solution/100g, intraperitoneal), and the common carotid artery exposed and placed within the Transonic flow probe (0.5 VB). Flow rate and heart rate were recorded simultaneously.

Circumferential vascular compliance was measured using a standard micromanipulator and microinjector (Nashige, IM 300). The left carotid artery was isolated, sutured closed at the distal end, and sealed proximally with suture onto a microneedle. The carotid artery was then inflated to 50, 100, 150, 200, and 250 mm Hg. Vessel diameter was measured from digitized images taken at each pressure (wild type: n=4, OPN null: n=4). Extensibility was calculated as described by Li et al.16 Example: extensibility at 125 mmHg = [(diameter at 150 mmHg)-(diameter at 100 mmHg) / (diameter at 100 mmHg)] x 100. A similar experiment was performed on a segment of the descending aorta between the renal arteries and the iliac bifurcation (wild type: n=4, OPN null: n=4). The isolated, segment was sutured closed at the proximal end, and sealed distally with suture onto a blunted 30 gauge needle. The aorta was then uniformly pressurized at a rate of 3ml/min using an Intraflow valve, with pressure measured with a standard arterial line pressure transducer. Longitudinal compliance of carotid arteries was determined similarly. The proximal end of the carotid artery was sutured closed, then tied to the inside of a petri dish filled with lactated Ringerís solution. The distal end was then sutured closed and tied so as to support a hanging weight. Vessel length was measured from digital photographs taken before and after application of the force.

Arterial ligation model

Ligation of the left carotid arteries of mice (wild type: n=14 at 28d, n=8 at 14d, n=8 at 4d, n=8 unmanipulated, n=6 sham; OPN null: n=14 at 28d, n=12 at 14d, n=8 at 4d, n=8 unmanipulated, n=6 sham) proximal to the internal/external carotid artery bifurcation was performed as previously described[91], with sham-operated mice used as controls. Bromodeoxyuridine injections (100µl of 25 mg/ml bromodeoxyuridine solution)

were given to each animal 15h and 1h prior to harvest. At the indicated times following ligation, tissues were perfusion fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.3. The perfusate was allowed to drain from an incision in the left carotid artery just proximal to the ligature. Vessels were harvested and embedded in paraffin. At least 800 consecutive serial sections were cut from both arteries of each mouse (7 μm thick). For the right carotid artery, all analyzed sections were cut from the vessel distal to the brachiocephalic artery.

Western blot analysis

Single ligated left carotid arteries were harvested and homogenized in lysis buffer (50mM TRIS pH=8.0, 0.1% SDS, 2.5mM NaCL). 100 mg total protein/sample was analyzed by SDS-PAGE (12.5% gel) followed by transfer to PVDF membrane, and immunoblotted using an anti-OPN antibody282 (2k1, kindly provided by Dr. T. Uede). Recombinant OPN was used as a positive control, and the relative amount of OPN/sample was quantified using Imagequant software and normalization to smooth muscle α-actin (clone 1A4, Sigma) levels. Triplicate analyses were performed.

Morphometric analysis

Morphometric analysis was carried out on ligated left common carotid arteries from both wild type and OPN null mice harvested at 14 and 28 days following ligation as well as unmanipulated and sham manipulated controls. The contralateral right common carotid artery was analyzed from harvests 28 days following ligation as well as unmanipulated and sham manipulated. Eight sections were collected in 280 mm intervals from the proximal 2.25mm closest to the ligature and orcein-stained for analysis (NIH

Image 1.60). The lumen surface, the perimeter of the neointima, and the perimeter of the tunica media were traced, yielding circumference and area of the lumen, internal elastic lamina, and external elastic lamina, respectively. Very small folds were not included in the IEL and external elastic lamina measurements. The medial area was calculated by subtracting the area defined by the internal elastic lamina from the area defined by the external elastic lamina. Intimal area was calculated as the area between the lumen surface and internal elastic lamina. As the shape of the cross sections is often distorted due to sectioning and the neointima may be unevenly distributed, lumen area could not be measured directly. Assuming the external elastic lamina was circular, lumen area was calculated by subtracting medial area and intimal area from the total area calculated from the circumference of the external elastic lamina. No difference in vessel areas was observed between sham and unmanipulated animals. Sections adjacent to the orcein-stained sections were stained with hematoxylin, and the number of nuclei in the media and intima for ligated, contralateral, and control arteries were counted using software developed specifically for this project by Sentient Machines (Wells, ME), which was validated to be consistent with manual counts. Lumen area of aortae of anesthetized unmanipulated mice was also measured in situ by magnetic resonance angiography (Siemens Magneton) utilizing blood flow for contrast (wild type: n=2, OPN null: n=2), and total artery size quantified by direct measurement following surgical exposure of the carotid artery (wild type: n=2, OPN null: n=2).

Immunostaining

A total of 8 sections, taken every 280 μm apart from each mouse harvested at 2 weeks following ligation were analyzed for cell proliferation using an anti-bromodeoxyuridine antibody (ICN Biomedicals) at a 1:2000 dilution followed by a biotinylated secondary antibody at a 1:500 dilution. Similarly, a total of 10 sections, taken every 280 μm apart from each mouse harvested at 4 days following ligation were analyzed for leukocyte infiltration using a biotinylated anti-CD45 antibody (ICN Biomedicals) at a 1:200 diluition. Sections were then treated with ABC reagent (Vector Labs.), followed by incubation with 3,3í-diaminobenzidine (Sigma). Digitized images of CD45 stained vessels were analyzed (NIH Image 1.60), and the staining quantified by first thresholding all non-stained areas from the image, then measuring the remaining positively stained area. Sections directly adjacent to those analyzed for leukocyte infiltration were analyzed for apoptosis using a TUNEL assay kit (Clontech, Apo-alert DNA fragmentation assay). Collagen was visualized with Massonís trichrome stain.

Statistical analysis

Morphometric data was structured as a two factor (OPN status, harvest time) randomized design then analyzed using Fisherís LSD procedure. Two factor ANOVA was performed on medial, intimal, lumen and total area measurements. Each showed OPN to have a significant effect. The effect of OPN was then further clarified using Studentís t-test. Remaining comparisons involving a single variable (OPN genotype) at a single time point were carried out with Studentís t-test. For the blood flow studies, measurements were considered outliers if they were greater than 3 standard deviations away from the

mean, and were excluded from analysis of blood flow rate. Most of the outliers were found to have heart rates lower than 250 beats/min, which is consistent with the observations of Hart et al. that heart rates below a certain point dramatically affect stroke volume 10. Thus, we considered flow measurements in mice with heart rates lower than 250 beats/min to be invalid, as flow rate correspondingly was decreased. All data are reported as mean ± standard deviation.

Chapter 3 Results

Vascular characteristics of OPN null mice

OPN null mice are overtly similar to wild type mice with respect to size, viability, and physical activity. Low levels of OPN expression in unmanipulated arteries have been previously described274. As the significance of this expression had not been explored, we first compared OPN null mice to wild type mice with respect to cardiovascular function. Blood pressure and heart rate measurements were obtained using the tail cuff method. Unexpectedly, we observed significantly lower blood pressure and increased heart rate in OPN null mice. Heart rate in the wild type group was 490 ± 57 beats/min, compared to 600 ± 62 beats/min in the OPN null group ($p< 0.001$)(Figure 10A). Conversely, the systolic blood pressure in wild type mice was 146 ± 12.2 mm Hg, compared to 130 ± 9.8 mm Hg in the OPN null mutants ($p< 0.001$)(Figure 10B). As increased heart rate often results from anemia, we analyzed blood cell composition by complete blood count (Table 5). No difference in red blood cells and platelets were observed, but a clear trend in increased white blood cells was observed in the OPN null mice. Further analysis showed that this WBC trend was accounted for by a significant increase in lymphocytes in OPN null mice ($p=0.033$).

We then sought to determine the consequences of altered heart rate and blood pressure on blood flow. A standard dosage of anesthesia resulted in equal heart rates in both OPN null (314 ± 40 beats/min) and wild type mice (320 ± 40 beats/min). Blood flow through the common carotid artery was determined by placing a flow probe around the exposed vessel. The blood flow in wild type mice was significantly greater than in OPN null mice at similar heart rates. Whereas the flow rate in the wild type vessels was 0.68 ± 0.16 ml/min, average flow in the OPN null vessels was 0.50 ± 0.21 ml/min ($p=0.029$)(Figure 10 C). These results suggest that the increased heart rate in OPN null animals may be due to a compensatory mechanism to counter reduced stroke volume. Blood flow is predicted to be similar in conscious mice due to the higher heart rate in OPN null mice.

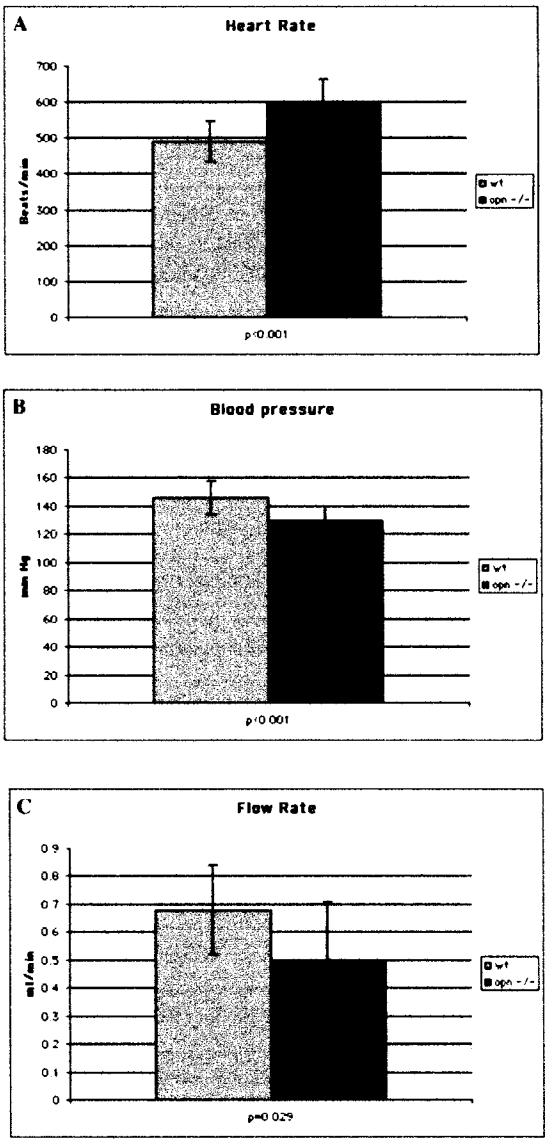


Figure 10. The loss of osteopontin results in altered hemodynamics. Though OPN null mice are similar to wild type mice with respect to appearance, activity tolerance, viability, and fertility, closer examination reveals a cardiovascular phenotype associated with the loss of OPN. As compared to wild type, OPN null mice demonstrate: (A) increased heart rate, (B) lower systolic blood pressure, and (C) decreased blood flow at equivalent heart rates.

	RBC	HGB	HCT	PLT	WBC
wild type	11.31+/- 0.62	18.36+/- 0.83	53.98+/- 2.92	1096+/- 120	4.22+/- 1.06
OPN null	11.10 +/-1.22	16.64+/- 2.72	53.28+/- 6.30	1273+/- 608	5.13+/- 0.09
p value	0.678	0.15	0.79	0.477	0.089
	NEU	LYM	MONO	EOS	BASO
wild type	0.77 +/- 0.22	3.30+/- 0.95	0.07+/- 0.06	0.08+/- 0.13	0 +/- 0
OPN null	0.66 +/- 0.20	4.37+/- 0.89	0.08+/- 0.07	0.01+/- 0.02	0 +/- 0
p value	0.305	*0.033*	0.614	0.154	ND

Table 5. The loss of osteopontin results in an altered concentration of circulating lymphocytes.

Comparison of complete blood count and differential cell counts of unmanipulated animals shows OPN null mice to have significantly more lymphocytes than wild type mice. No significant difference in other values was noted. Values presented are average ± S.D. The p value listed represents a comparison in each population between the wild type and OPN null values. RBC: red blood cells, HGB: hemoglobin, HCT: hematocrit, PLT: platelets, WBC: white blood cells, NEU: neutrophils, LYM: lymphocytes, MONO: monocytes, EOS: eosinophils, BASO: basophils, ND: not defined. Italics indicate statistical significance.

To determine if the vessel structure of OPN null mice reflected the altered hemodynamics, we compared magnetic resonance angiograms of OPN null mice to wild type. No difference was observed in aortic lumen areas (Figure 11). To expand on the angiograms, digital photographs of surgically exposed common carotid arteries were compared and also showed no difference in total vessel size. Having found no difference in arterial morphology, we suspected that characteristics of the arterial wall were related to the rate of blood flow and heart rate, and thus measured carotid artery wall circumferential compliance as a function of intravascular pressure. Determination of small vessel circumferential compliance has been previously described[31]. Left carotid arteries were isolated, then inflated to specific pressures. Vessels were digitally recorded at pressures from 50 mm Hg to 250 mm Hg. In OPN null mice, we observed a very rapid increase in vessel wall diameter at physiological pressures (as determined by our blood pressure measurements of these mice), while carotid arteries from wild type mice demonstrated similar increases in size only at significantly higher pressures (Figure 12). The percentage increases in vessel size at all pressures measured was greater in vessels from OPN null mice, compared to wild type mice, and the responses of each carotid artery sample of a particular genotype was remarkably consistent. To exclude the possibility that increased circumferential compliance in OPN null mice was unique to the carotid artery, a similar experiment was performed on segments of abdominal aorta (Figures 13,14). The aorta demonstrated a similar increase in circumferential compliance in OPN null animals as compared to wild type. Interestingly, the compliance data predicts OPN null arteries to be larger than wild type at physiological pressures, which is not observed in situ. We interpret this apparent inconsistency as supportive evidence of increased vascular tone in OPN null mice as a compensatory mechanism for increased

compliance. Circumferential compliance is mediated by both elastin and collagen content, whereas longitudinal compliance is mediated predominantly by elastin[13,18]. To determine if longitudinal compliance was altered from that of wild type in OPN null mice, a left common carotid artery from each group was mounted in a petri dish so as to support different weights along the longitudinal axis. No difference in longitudinal compliance was observed (Figure 15).

As the compliance data suggested collagen might be altered in OPN null mice, we examined vessels histologically in an effort to determine the origin of the difference in compliance. Arterial cross sections were stained with orcein and Massonís trichrome stain to visualize elastin (Figure 16A,B) and collagen (Figure 16C,D), respectively. No difference in the elastic fibers was noted between groups, but the collagenous matrix appeared to be organized much more loosely in the OPN null mice, particularly in the adventitial region. Interestingly, this observation does not correlate with the observation of Giachelli et al. which localized the major sites of OPN expression to along the elastic laminae[274].

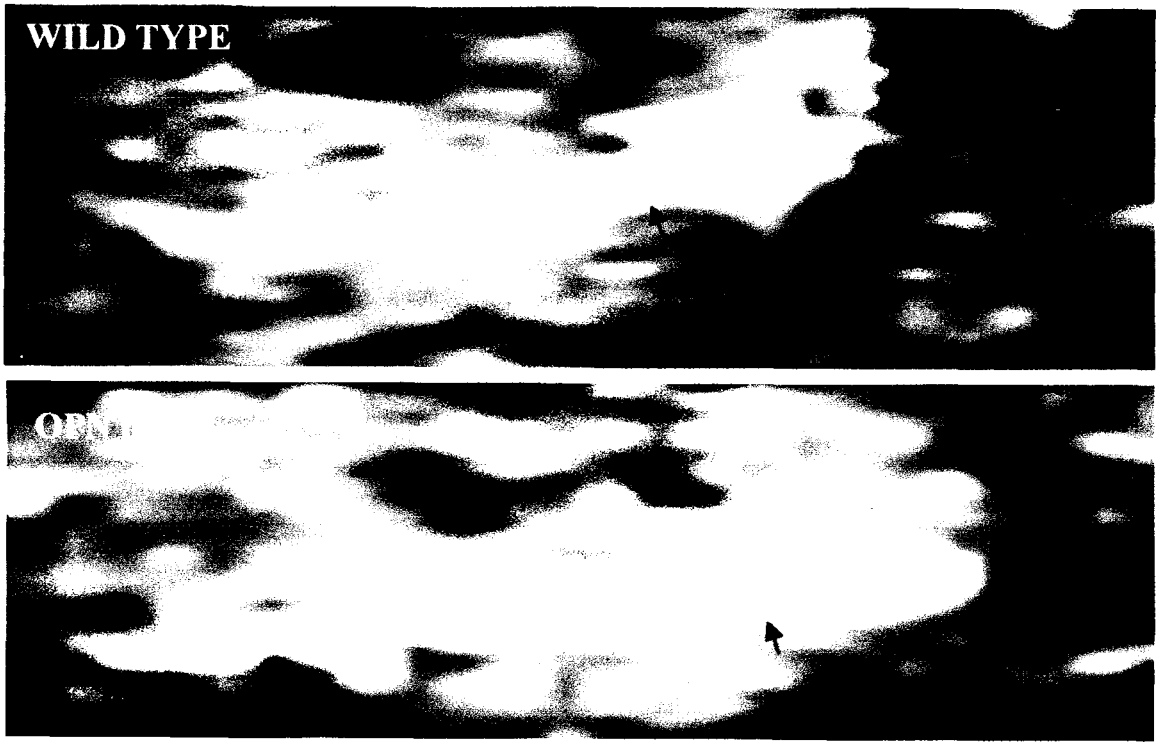


Figure 11. Magnetic resonance angiograms show the aorta of OPN null mice to be the same size as wild type mice. Mice (Wild type: n=2, OPN null: n=2) were anesthetized with avertin and examined in a magnetic resonance imaging scanner intended for human use. As the diameter of the aorta varies along its length, only a single measurement from a comparable location (indicated by arrow) was made from each mouse. Despite the poor resolution, the aorta diameter of OPN null mice (n=2) can be seen to be not significantly different than that of wild type (n=2).

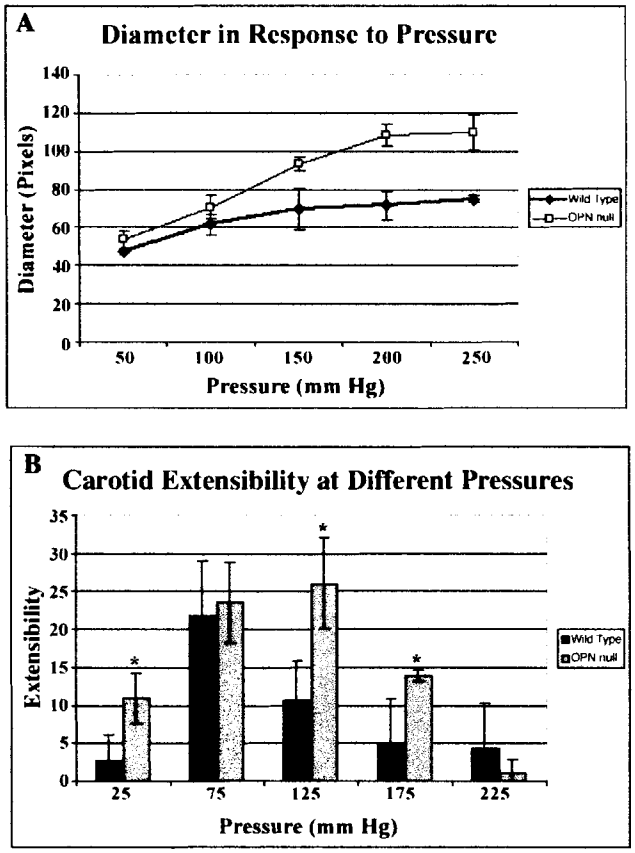
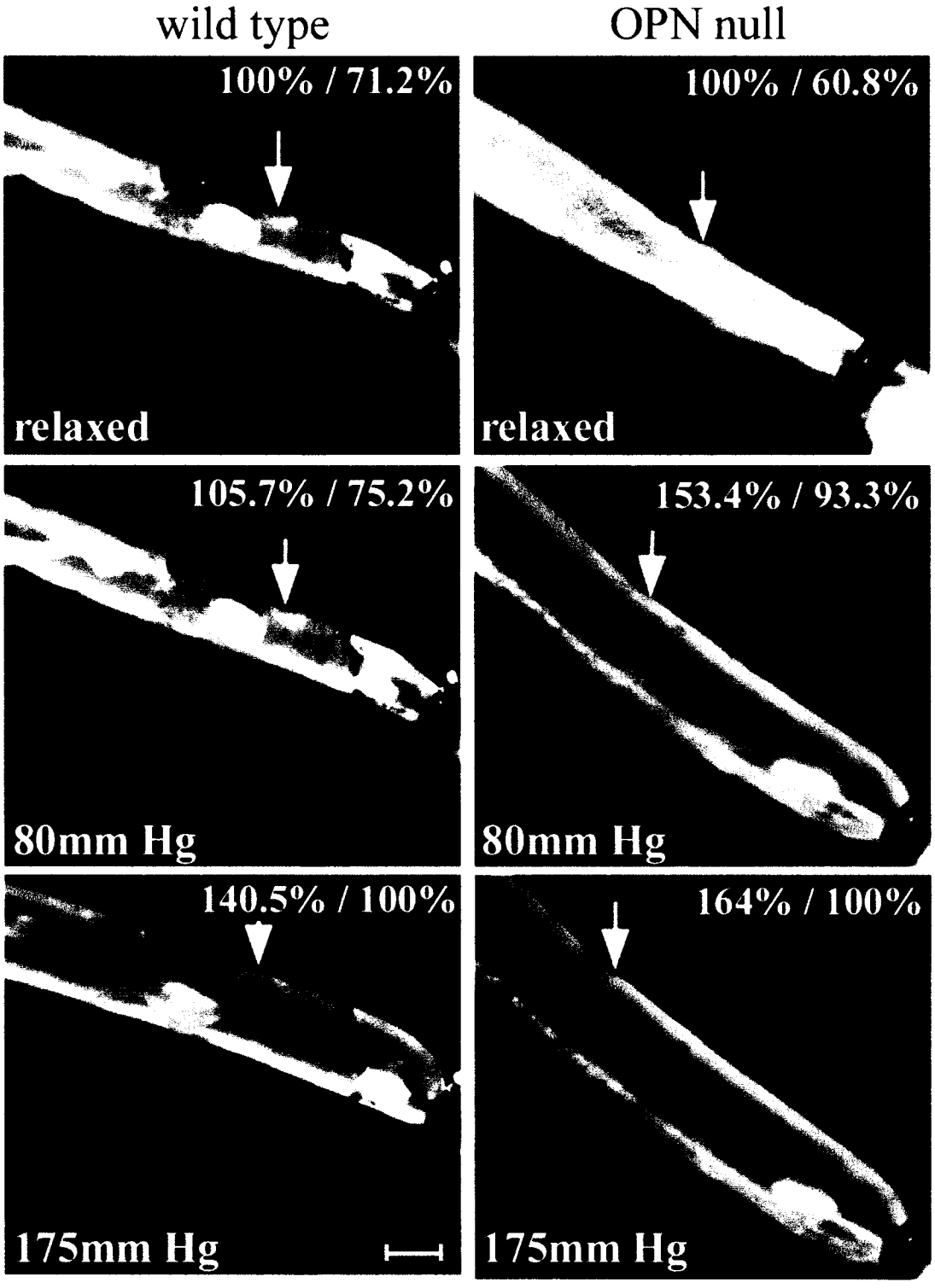


Figure 12. The loss of osteopontin results in the increased arterial wall circumferential compliance of the common carotid artery. Left carotid arteries were isolated from wild type and OPN null mice, then sutured to a catheter such that intravascular pressure could be regulated. Intravascular pressure was slowly increased to allow equalization. Diameters were then measured from digitized images taken at the indicated pressures. (A) OPN null carotid arteries demonstrate greater compliance than wild type at physiological pressures. Note that the increase in diameter of OPN null vessels is greater than that of wild type. (B) Measurements of vessel diameter at the corresponding pressure were used to determine vessel extensibility. The carotid arteries of OPN null mice demonstrate greater extensibility than wild type at the pressures determined in earlier experiments to be physiological for these mice. Asterisks (< 0.05).

Figure 13. The loss of osteopontin results in the increased arterial wall circumferential compliance of the aorta. Segments of aortae extending from the renal artery to the iliac bifurcation were isolated from wild type and OPN null mice, then sutured such that intravascular pressure could be modulated / measured with standard arterial line equipment. Arterial segments were then inflated with isotonic saline at 3 ml/min. Digitized photographs were taken at indicated pressures. As the diameter of the aorta differs along its length, only a single measurement from a comparable location was made from each mouse. Arterial diameter was measured at sites indicated by arrows. Indicated in each panel is vessel size as a percentage of the relaxed vessel and vessel size as a percentage of the expanded vessel at 175 mm Hg. OPN null mice demonstrated greater arterial wall compliance than wild type mice as indicated by a greater increase in vessel diameter at both 80 mm Hg (wild type: 106% of relaxed diameter, OPN null: 153% of relaxed diameter) and 175 mm Hg (wild type: 141% of relaxed diameter, OPN null: 164% of relaxed diameter). Bar = 2mm.


wild type
OPN null
100% / 71.2%
relaxed
100% / 60.8%
relaxed
105.7% / 75.2%
80mm Hg
153.4% / 93.3%
80mm Hg
140.5% / 100%
175mm Hg
164% / 100%
175mm Hg

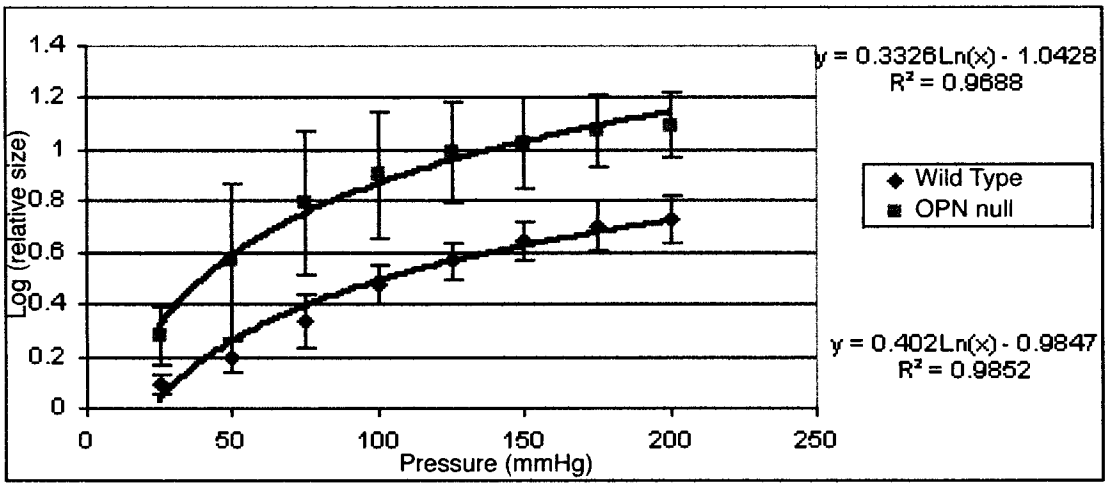


Figure 14: The aortae of OPN null mice demonstrate increased compliance. Aortae were isolated from wild type and OPN null mice, then sutured to a microinjector such that intravascular pressure could be regulated. Diameters were then measured from digitized images at indicated pressures. Regression formulae and associated R2 describe data for both wild type (top) and OPN null mice (bottom)

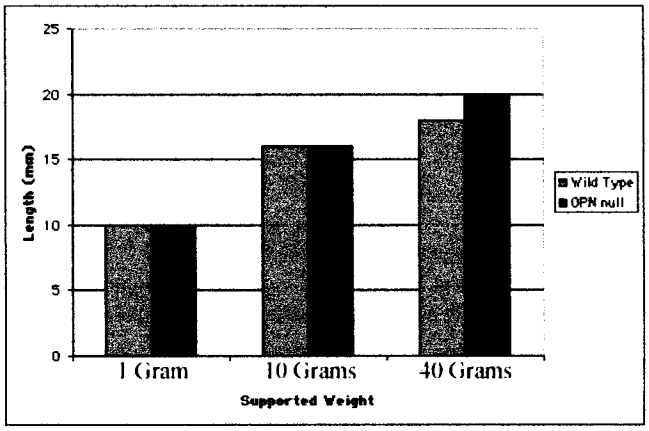


Figure 15. The presence of osteopontin does not affect the longitudinal compliance of the common carotid artery. Left common carotid arteries of equal length from a wild type (n=1) and an OPN null mouse (n=1) were mounted in a petri dish so as to photograph the vessels before and after application of a known force along the longitudinal axis. No difference in vessel length was observed between wild type and OPN null mice.

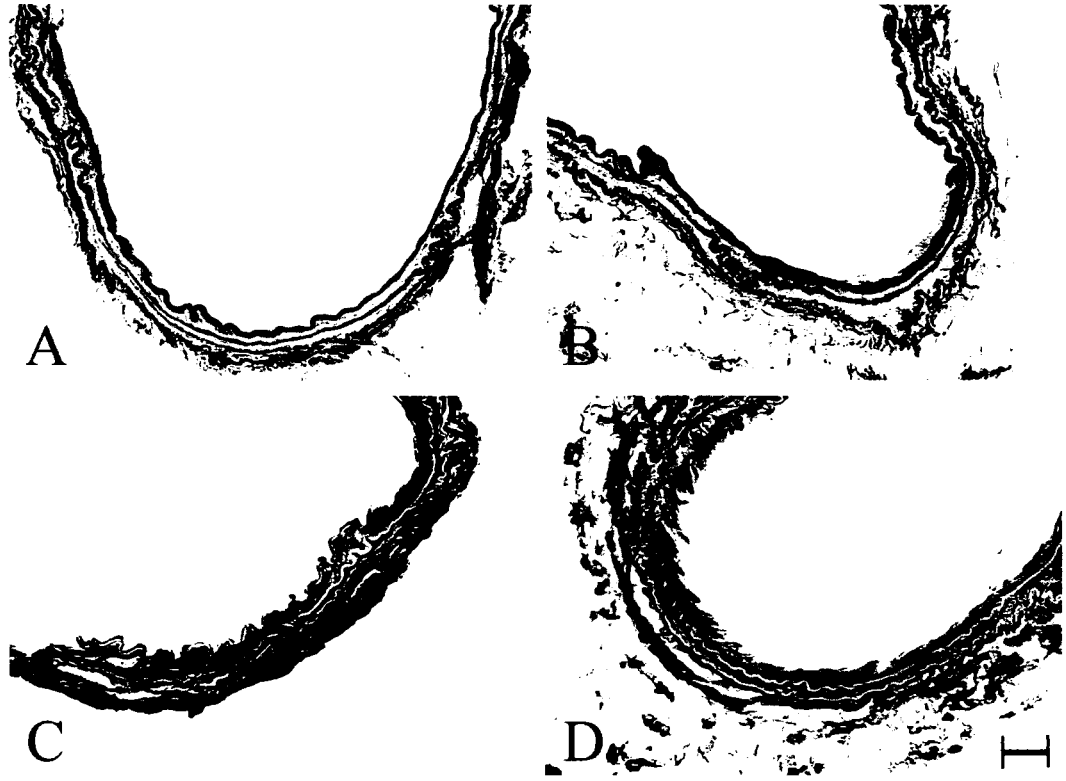


Figure 16. Collagen fibers are organized more loosely in vessels of OPN null mice. Sections from unmanipulated carotid arteries were stained with orcein (A, B) or Massonís trichrome stain (C, D) to visualize elastin and collagen, respectively. No difference in organization of elastin fibers was noted in wild type (A) versus OPN null vessels (B). However, the organization of adventitial collagen fibers in arteries from OPN null mice (D) appeared looser compared to wild type arteries (C). Elastin appears brown in A and B, collagen appears blue in C and D. Bar=20

m□m .

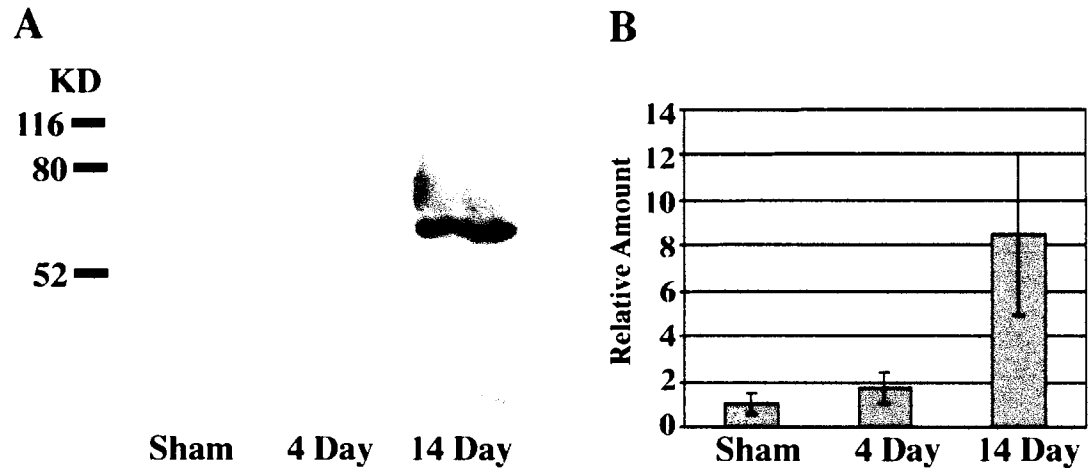


Figure 17. Osteopontin expression is upregulasted following carotid artery ligation. (A) Western blot of 100 mg of total protein from individual carotid arteries was performedwith 12.5% SDS-PAGE followed by blotting with anti-OPN. (B) OPN bands were then quantified following normalization against smooth muscle α-actin. The blot was repeated in triplicate and results are reported as mean with standard deviation.

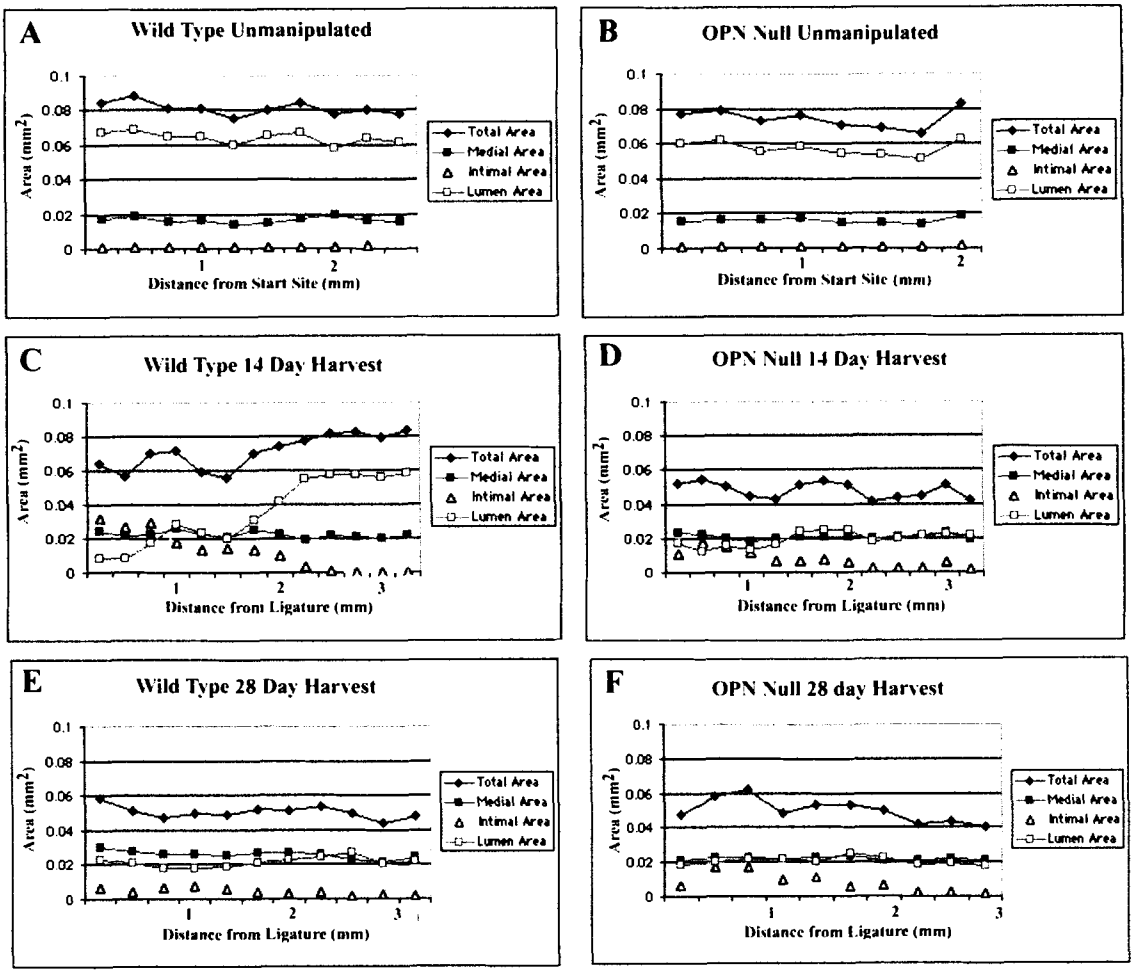
Expression of OPN following blood flow cessation in carotid arteries

To quantify the temporal induction of OPN protein following carotid artery ligation, vessels from OPN null or wild type mice were collected at 4 and 14 days after ligation. Sham manipulated vessels were collected as control samples. Single ligated left carotid arteries from each group were homogenized, then analyzed by Western blot for the presence of OPN (Figure 17A,B). OPN protein in each vessel was quantified by densitometric comparison and normalized to smooth muscle α-actin levels. Antibodies to smooth muscle α-actin was used instead of antibodies to smooth muscle β-actin in an effort to normalize the length of the arterial segment analyzed (proliferation of cells

following ligation would be expected to increase the amount of β-actin present as compared to an equivalent length segment of uninjured artery. We sought to determine the differential expression of OPN in arterial segments of equivalent length, as opposed to the number of cells present.) In vessels from wild type mice, low levels of OPN were detected in unmanipulated carotid arteries. OPN protein was increased at 4 days after ligation, and was abundant at 2 weeks after ligation. No OPN was detected in any arterial samples from OPN null mice.

Vascular remodeling response is regulated by OPN

We used the carotid artery ligation model to test the hypothesis that OPN regulates vascular remodeling responses. Groups of wild type and OPN null mice were ligated with sham manipulation and unmanipulated mice as control. Carotid arteries were then harvested at 14 and 28 days following ligation. We chose to harvest the carotid arteries at 14 and 28 days following the procedure as previous work has indicated these times to be optimal for proliferation92, and a time of stable neointima formation91 respectively. Eight sections from the proximal 2.25mm closest to the ligature were analyzed from each mouse (Figure 18). No differences in vessel areas were found between unmanipulated and sham manipulated arteries (Table 6). Consistent with their increased compliance upon isolation from the body, the unmanipulated carotid arteries in OPN null mice were significantly smaller than those in wild type counterparts (Table 6). Following ligation, the ligated left carotid artery in all mice showed negative remodeling (decrease in total area), while the contralateral right artery underwent positive remodeling (increase in total area).


A
Wild Type Unmanipulated
Area (mm²)
0.1
0.08
0.06
0.04
0.02
0
1
2
Distance from Start Site (mm)
Total Area
Medial Area
Intimal Area
Lumen Area
B
OPN Null Unmanipulated
Area (mm²)
0.1
0.08
0.06
0.04
0.02
0
1
2
Distance from Start Site (mm)
Total Area
Medial Area
Intimal Area
Lumen Area
C
Wild Type 14 Day Harvest
Area (mm²)
0.1
0.08
0.06
0.04
0.02
0
1
2
3
Distance from Ligature (mm)
Total Area
Medial Area
Intimal Area
Lumen Area
D
OPN Null 14 Day Harvest
Area (mm²)
0.1
0.08
0.06
0.04
0.02
0
1
2
3
Distance from Ligature (mm)
Total Area
Medial Area
Intimal Area
Lumen Area
E
Wild Type 28 Day Harvest
Area (mm²)
0.1
0.08
0.06
0.04
0.02
0
1
2
3
Distance from Ligature (mm)
Total Area
Medial Area
Intimal Area
Lumen Area
F
OPN Null 28 day Harvest
Area (mm²)
0.1
0.08
0.06
0.04
0.02
0
1
2
3
Distance from Ligature (mm)
Total Area
Medial Area
Intimal Area
Lumen Area

The ligated left carotid artery of OPN null mice showed significantly less neointima formation and greater constrictive remodeling (as evidenced by change in total area) at 14 days following ligation compared to that of wild type mice (Table 6). Interestingly, the negative remodeling occurred such that the lumen area was no longer different between groups. At 28 days following ligation, additional remodeling resulted in similar intimal, lumen, and total areas between wild type and OPN null mice. At this time, only medial area demonstrated a significant difference, and was smaller in the OPN null than wild type vessels. Thus, the carotid arteries of OPN null mice displayed different remodeling responses to arrive at 28 day remodeled arteries that were morphologically similar to controls.

The right common carotid artery of all mice showed compensatory increases in lumen area ($p<0.001$) and total area ($p<0.001$) with no significant changes in medial or intimal areas as compared to unmanipulated vessels (Table 7). Consistent with having increased compliance, the actual change in lumen area was greater in OPN null mice.

Regression analysis describes osteopontin effects on the response to carotid artery ligation in greater detail

Having demonstrated the loss of OPN to alter the vascular injury response, we sought to determine if OPN affected how the injury response changed with distance from the site of ligation. Using the analytical method described in chapter 2, we found the response of OPN null mice to be dramatically different than that of wild type mice. Whereas wild type mice showed changes in vessel areas with increasing distance from the site of ligation, OPN null mice did not. Additionally, the changes in vessel areas that characterized the wild type response changed with harvest time. Though OPN null mice

Medial Area **Intimal Area**

	Control	14d	28d	Control	14d	28d
Wild Type	0.016 +/- 0.002	0.023 +/- 0.005	0.027 +/- 0.006	NA	0.02 +/- 0.024	0.017 +/- 0.033
OPN Null	0.016 +/- 0.002	0.021 +/- 0.007	0.023 +/- 0.005	NA	0.005 +/- 0.003	0.015 +/- 0.022
P(wt/null)	0.375	*0.040*	*<0.001*		*0.003*	0.659

Lumen Area **Total Area**

	Control	14d	28d	Control	14d	28d
Wild Type	0.064 +/- 0.008	0.022 +/- 0.018	0.019 +/- 0.001	0.081 +/- 0.009	0.065 +/- 0.032	0.061 +/- 0.025
OPN Null	0.057 +/- 0.008	0.019 +/- 0.013	0.024 +/- 0.019	0.074 +/- 0.009	0.050 +/- 0.025	0.061 +/- 0.032
P(wt/null)	*0.002*	*0.420*	*0.087*	*0.005*	*0.024*	0.785

Table 6. Traditional morphometric analysis of the ligated left carotid artery.

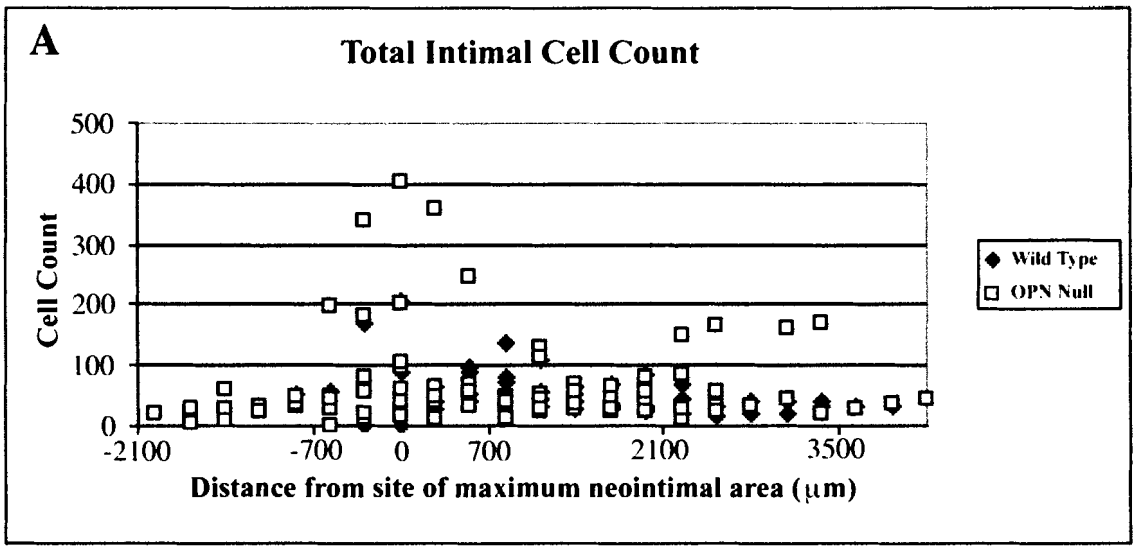
Comparison of the injury response of OPN null mice to wild type mice was first done with mean areas for the entire vessel as described by Kumar and Lindner91. Mean medial, intimal, lumen, and total areas from left carotid arteries harvested 14 and 28 days following left carotid artery ligation, and from left carotid arteries harvested from unmanipulated mice are presented. Data from sham manipulated mice did not differ from unmanipulated at any time point. Two way ANOVA (OPN/harvest time) indicated OPN to have a significant effect that was then clarified with Studentís t tests. At 14 days following ligation, OPN null mice demonstrated greater constrictive remodeling (as indicated by a decrease in total area) and less neointima formation than wild type mice. At 28 days following ligation, with the exception of medial area, the remodeled arteries of OPN null mice did not differ from wild type. Values presented are average areas (mm2) ● S.D. NA: values too small to be precisely measured. Italics indicate statistical significance.

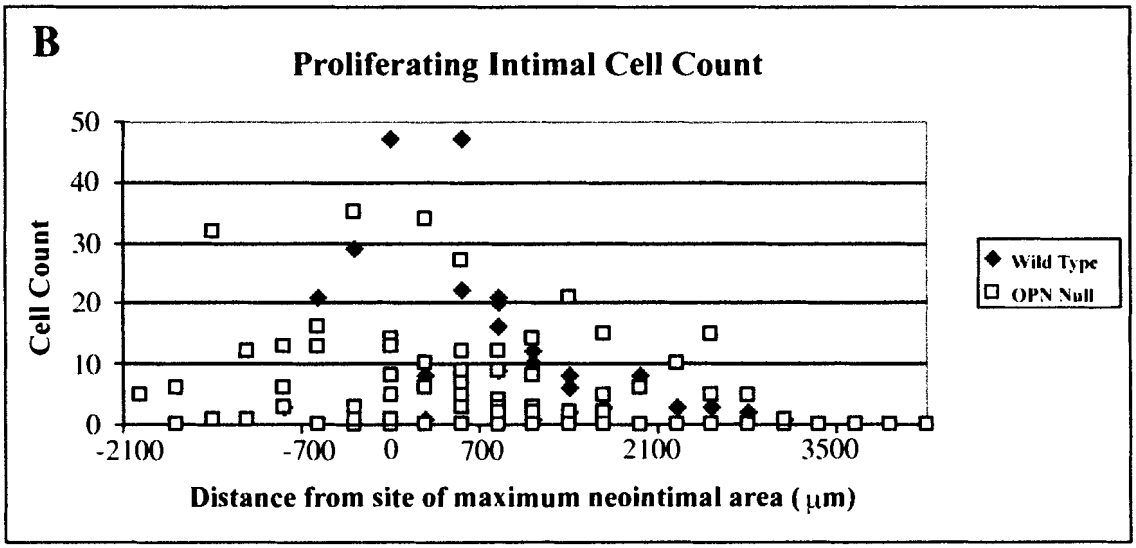
Source	Media	Intima	Lumen	Total
OPN (before injury)			x	x
Distance (before injury)	x	x	x	
OPN (after ligation)		x	x	x
Distance (after ligation)				
Harvest time(after ligation)	x	x	x	x
OPN * Harvest (after ligation)		x	x	
OPN * Distance (after ligation)		x	x	x
OPN * Harvest * Distance (after ligation)	x	x	x	x
R2	0.5105	0.2145	0.3907	0.3610

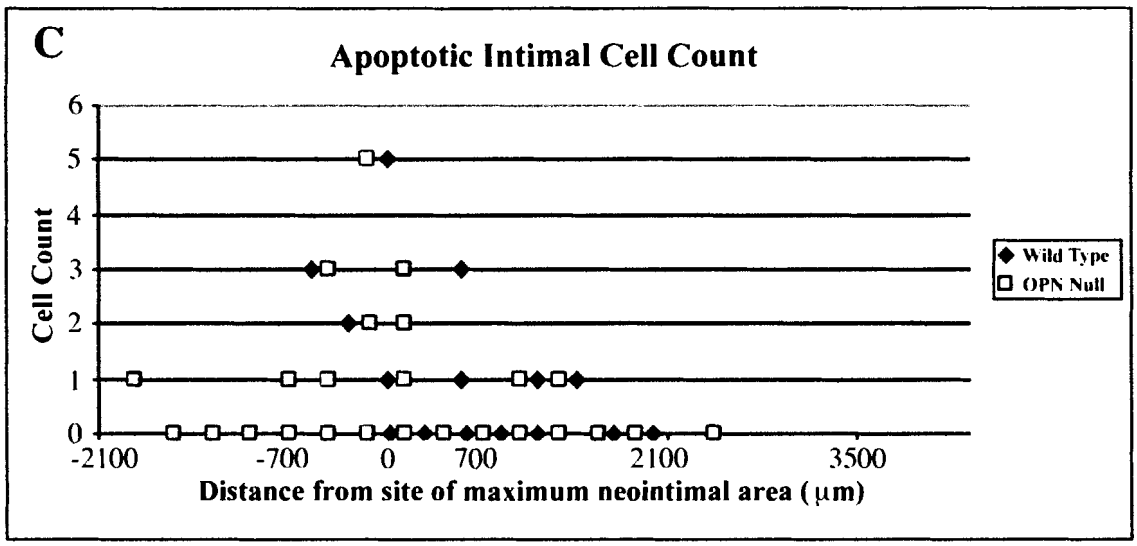
Table 8. Regression models describe how osteopontin is affecting the response to carotid artery ligation. Though the response to ligation changed with harvest time in both wild type and OPN null mice, the response of wild type mice showed vessel area changes with distance that were not constant at each harvest time. Checked boxes indicate the associated parameter was found to be statistically significant ($p<0.05$).

Cell proliferation and apoptosis

Our morphometric analysis demonstrated that the loss of OPN does not affect remodeling responses along the vessel in a uniform manner. We sought to determine if the observed difference in neointima formation at 14 days between wild type and OPN null mice could be explained by altered cell proliferation or apoptosis. Sections directly adjacent to those analyzed for morphology were used to determine intimal cell number. At 14 days following ligation, no difference was observed in the total number or distribution of intimal cells (Figure 19A). Total cell number was observed to correlate with intimal area. Sections were additionally analyzed for proliferation by bromodeoxyuridine incorporation. Again, no difference was observed in total number of proliferating cells (Figure 19B). As with total cell number, cell proliferation correlated with intima area with approximately 10% of intimal cells undergoing proliferation. Apoptotic cells were quantified on adjacent sections using a fluorescent deoxyribonucleic acid end labeling assay (Figure 19C). In both wild type and OPN null mice, apoptosis was observed in only 1% of the intimal cells. Like cell proliferation, the presence of intimal apoptotic cells was observed to correlate with intimal area. While these particular experiments do not indicate differences on the OPN null background, the results demonstrate that the size of the neointima correlates with high rates of cell turnover. Thus, the injury response at the cellular level also demonstrates regional differences.


A
Total Intimal Cell Count
500
400
300
200
100
0
Cell Count
-2100
-700
0
700
2100
3500
Distance from site of maximum neointimal area (μm)
Wild Type
OPN Null


B
Proliferating Intimal Cell Count
50
40
30
20
10
0
Cell Count
-2100
-700
0
700
2100
3500
Distance from site of maximum neointimal area (μm)
Wild Type
OPN Null


C
Apoptotic Intimal Cell Count
6
5
4
3
2
1
0
Cell Count
-2100
-700
0
700
2100
3500
Distance from site of maximum neointimal area (μm)
Wild Type
OPN Null

Rich inflammatory infiltrate following carotid artery ligation

As OPN has been shown to control the influx of inflammatory cells in vivo, we examined if early inflammation was quantitatively different between OPN null and wild type animals. Using an antibody against the pan-leukocyte marker CD45, immunostaining was performed on sections of carotid arteries from both groups collected 4 days after ligation. In sections from wild type animals, CD45 positive leukocytes were abundant in the region with the largest neointima, but rare in the remainder of the vessel (Figure 20A). Analysis of OPN null animals revealed few leukocytes in any region along the entire length of the carotid artery (Figure 20B). Quantification of the area of positive immunostaining of wild type mice showed a 10-fold increase over that of OPN null mice. We attempted to specify the type and activation state of the invading inflammatory cells using antibodies to CD-4, CD-8, CD-25, and CD-45 with flow cytometry. A digest time could not be found that allowed cell dissociation, and cell viability.

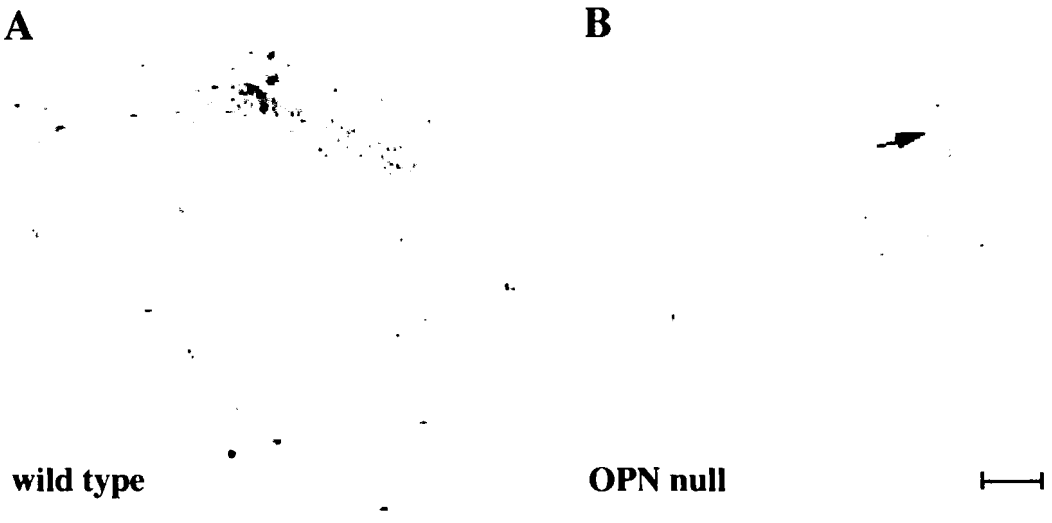


Figure 20. Leukocyte infiltration is diminished in OPN null mice. Representative section from wild type (A) and OPN null (B) vessels harvested 4 days following ligation in regions close to the ligature are shown. Sections were stained with anti-CD45 pan-leukocyte antibody. Note the absence of leukocytes in the OPN null sections. Arrow in panel B shows a region of some leukocyte influx. Bar = 500 μm.

Chapter 3 Discussion

Both gene products and physical forces have been shown to affect vascular physiology / pathophysiology. In this study, the loss of OPN was shown to alter hemodynamics and arterial mechanics. As normal wild type mice express very little OPN in blood vessels, our findings of significant changes in normal blood vessel physiology on the OPN null background was unanticipated. Compared to wild type, we observed unmanipulated OPN null mice to have more lymphocytes, lower systolic blood pressure, increased heart rate, arteries with greater compliance, and reduced blood flow at similar heart rates. These observations are consistent with previous work which has observed increased systolic pressure associated with stiffened vessels[22-24]. Though increased compliance can increase the lumen area of resistance vessels thus reducing peripheral resistance / blood pressure, other factors must be considered as this has not been observed in every case[31,50,70]. However, contrary to the predictions of Campbell[21] (Cardiac output = mean arterial pressure x (compliance / time constant)), we observe increased compliance in the presence of decreased cardiac output, suggesting the lowered systolic pressure observed in the OPN null mice of this study may reflect lowered mean arterial pressure. Interestingly, the difference in large artery diameters predicted by our compliance data was not observed in an in situ comparison of OPN null mice to wild type. Though our in situ observations were not in alert mice (and we did not measure the blood pressure of the anesthetized mice), we interpret this inconsistency as supportive evidence of a compensatory increase in vascular tone in OPN null mice.

Our data support the following model of OPN activity in the normal vessel wall (Figure 21). As OPN null mice are similar to wild type in size, physical activity, and oxygen carrying capacity (Table 5, and data not shown), blood flow is similar in both

groups. Because of the lack of OPN, vessel wall compliance is greater, resulting in decreased systolic pressure. Though the OPN null mice attempt to compensate for increased compliance with increased smooth muscle cell tone, this compensation is incomplete as evidenced by lower blood pressure [Arterial pressure=(flow)(vascular resistance)]. Additionally, the loss of OPN is associated with reduced stroke volume which is compensated for by an increased heart rate [Flow=(stroke volume)(heart rate)]. Although the OPN null mice are overtly similar to wild type mice, our model predicts the mice to be compromised upon cardiovascular challenge.

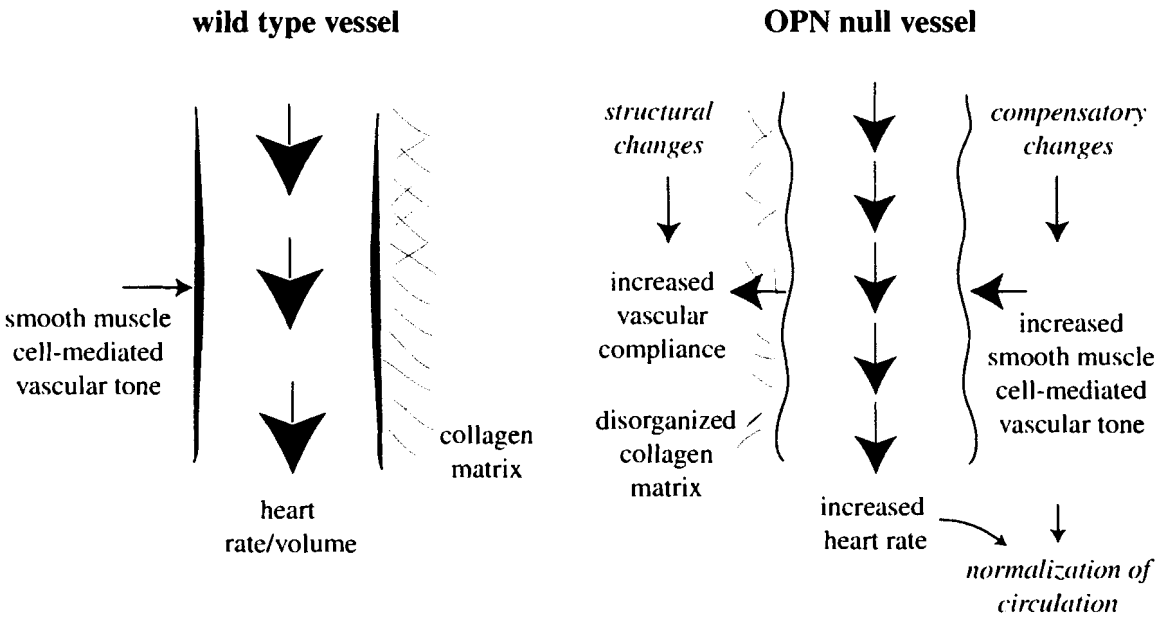


Figure 21. Model of altered hemodynamics in OPN null mice. The structural changes noted on the OPN null background include disorganized collagen and increased vessel wall compliance. OPN null mice compensate for increased vascular compliance with increased vascular tone, and reduced stroke volume with increased heart rate.

The structural change involving the collagen matrix is of interest, as OPN is thought to interact with collagens[186,201], fibronectin[202], and other components of specialized matrices including osteocalcin[174]. With respect to collagen, we previously found that on the OPN null background, wound healing in the skin occurred with a defect in the fibrillar organization of collagen, and formation of large dermal collagen fibrils was lacking[281]. Similarly, following myocardial infarction, Trueblood et al. observed increased dilation in the presence of smaller and more loosely organized collagen fibers in the remodeled heart of OPN null mice as compared to wild type[283]. In arteries, the circumferential stretch of arteries has been shown to mostly involve elastin and collagen structure[13,18,284]. Functionally, defective collagen organization has been shown to increase aortic compliance and reduce strength[19], which is consistent with our observations. Collectively, these observations suggest that the presence of OPN is important for matrix remodeling and collagen structure in vivo, and suggests that OPN modification of collagen structure may be the molecular mechanism underlying the altered compliance of OPN null mice.

As we had hypothesized, we found that the remodeling response of OPN null vessels to cessation of blood flow was distinct from that in wild type vessels. The response to carotid artery ligation has been shown to be mediated by factors affecting inflammation[101], vascular tone[27,102,118], plasma proteins[84,92,100,114], cytoskeletal components[32], hormones / growth factors[130,135]. Following ligation, OPN null mice demonstrated greater initial constrictive remodeling and less neointima formation as compared to wild type. The observation of decreased neointima formation in the response

of OPN null mice to carotid artery ligation is consistent with our previous observation of decreased neointima formation following endothelial denudation in rats treated with anti-OPN antibody[280]. As it was possible that the increased constrictive remodeling reflected an endothelium dependent response, we re-examined our earlier data using anti-OPN antibody infusion following balloon catheter denudation[280]. Similarly, we found significantly increased constrictive remodeling in the anti-OPN treated group compared to wild type (Figure 22). This suggests that blocking OPN function may be a means to increase vascular compliance. Another consistent finding between the endothelial denudation model and the carotid artery ligation model was that we could not detect changes in cell proliferation, apoptosis, or cell density.

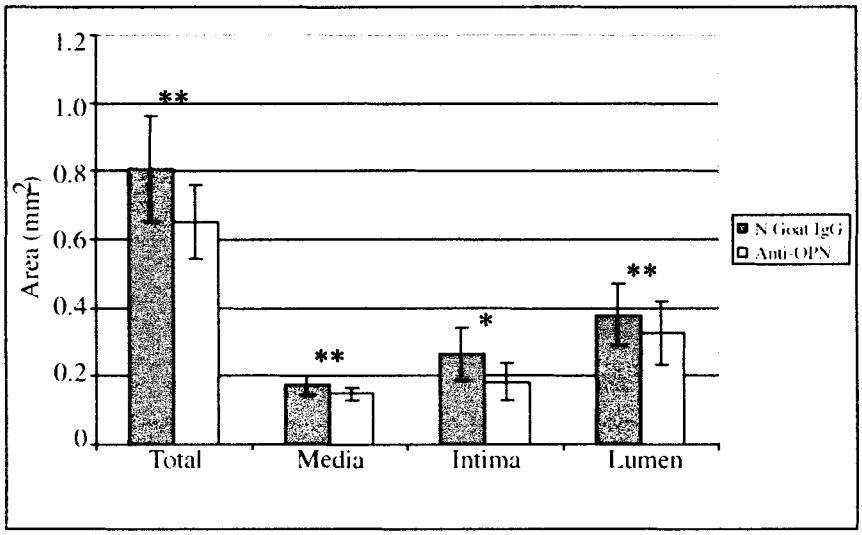


Figure 22. Following endothelial denudation, rats demonstrate less constrictive remodeling in response to anti-OPN treatment. Previously published data concerning the effect of OPN on the vascular remodeling of rats following endothelial denudation was reexamined. Consistent with the data presented in this chapter, the loss of OPN resulted in decreased constrictive remodeling. Single asterisk indicates a previously described significant difference. Double asterisks indicate significant difference found on review.

OPN null mice, despite having more circulating lymphocytes, did not demonstrate the early leukocyte infiltration that characterized the response of wild type mice. OPN has been verified as an important mediator of immune cell migration and activity[285] and leukocyte invasion has been observed to be an important determinant of neointima formation. For example, decreased leukocyte infiltration was associated with reduced neointimal size in P-selectin null mice[109] and endothelial nitric oxide synthase transgenic mice[102]. Conversely, endothelial nitric oxide synthase deficient mice demonstrated greater neointima formation in the presence of increased leukocyte infiltration[27] and fas ligand deficient mice showed increased neointima formation preceded by increased leukocyte infiltration[168]. While our study supports a positive association of leukocyte infiltration and neointimal lesion size, we also observed increased constrictive remodeling associated with decreased neointimal lesion size in OPN null mice. Interestingly, this altered remodeling occurred in such as a way to maintain the remodeled lumen size. The mechanism of the altered response will require additional study. While we hypothesized that loss of the macrophage chemoattractant properties of OPN would directly alter the vascular injury response of OPN null mice, the altered response may be secondary to the unexpected difference in hemodynamics/vessel mechanics. This alternative explanation is supported by several studies that have observed an association of inflammation with increased blood pressure[242,286].

As hypertension has been observed with increased calcification[243,287], and Speer et al.[278] observed increased induced arterial calcification in mice on an OPN null / Gla null

background, our observation of decreased blood pressure and increased compliance in OPN null mice initially seemed counterintuitive. It is most likely that the altered hemodynamics / vascular mechanics of OPN null mice are due to both primary and secondary effects of loss of OPN. We suspect the loss of direct inhibition of calcification by OPN overcomes the secondary feature of lower blood pressure. Thus, our observations rather than conflicting with those of Speer et al. combine to demonstrate the many factors that must be considered when analyzing vascular response.

In summary, the regulation of vascular function is indeed complex. This study demonstrated OPN to have a role in determining the hemodynamics / vessel mechanics of normal arteries as well as remodeled vascular phenotypes. Our data, combined with that of others, suggests alteration of collagen structure may be the mechanism by which compliance is changed in OPN null mice. We are currently seeking to distinguish which of the observed effects are primary and secondary due to loss of OPN.

Chapter 4

SUMMARY, FUTURE PLANS, AND RESEARCH DESIGN

Summary

The work presented in this text makes a significant contribution not just to the study of chronic obstructive vascular disease, but to the general study of vascular biology as it identifies OPN as not only having a role in vascular injury response, but in the function of normal, healthy arteries as well. Comparison of the morphology of the carotid arteries of wild type mice to OPN null mice before and after vascular injury demonstrated that OPN does not affect the morphology of large arteries before vascular injury or after stable lesion formation, but does affect the path by which the stable lesion is achieved. Comparison of the vascular function of large arteries between wild type and OPN null mice demonstrated the hemodynamics of uninjured OPN null mice are different than wild type mice, and the physical / structural characteristics of the large arteries are different as well. Comparison of the complete count of OPN null mice to that of wild type mice revealed OPN null mice to have a greater concentration of lymphocytes. The loss of OPN was shown to result in decreased inflammation and followed by less neointima formation. Additionally, the work demonstrated the first application of regression to the analysis of carotid artery ligation data that, due to increased comparative power and quality of description, should facilitate future experimentation and interpretation of studies. The work was exciting to perform as the differences between uninjured wild type and OPN null were not expected.

The work presented in this text has several limitations that prevent further interpretation of the results as they relate to the projectís goal, the determination of the mechanism by which OPN affects the vascular injury response. The major problem is the presence of OPN caused differences in the negative controls for the vascular response (sham manipulated and unmanipulated mice). Though this observation is exciting as it

demonstrates the first vascular phenotype of uninjured OPN null mice, and suggests that OPN may be a novel target for blood pressure therapy, the lack of equivalent baseline complicates analysis. The difference observed in the vascular injury response of OPN null mice from that of wild type mice may be due to conditions that existed prior to injury. This limitation occurred because we simply did not expect the small amount of OPN present in the vessel prior to injury to have a significant effect on vascular function. In retrospect, in vivo systems are complex, and the action of OPN at sites other than the vasculature must be considered as it may have secondary effects on the vasculature. For example, it is possible that the expression and action of OPN in regions of kidney involved with blood pressure regulation affected hemodynamics such that the structure of the vasculature compensated with altered compliance. Additionally, as inflammation has been shown to correlate with blood pressure[242], the diminished inflammatory response of OPN null mice may be secondary to reduced blood pressure. We chose to complete the study with the belief that the full data would help us design better follow-up experiments, and any follow-up experimental design would require the data.

A second limitation of the data that prevents further interpretation is the complete lack of data on vascular function after injury. Thus, the effect of vascular injury on hemodynamics and vascular compliance is not known. As expression of OPN increased following injury and a difference in injury response was observed at this time, the simultaneous observation of an effect of OPN on either compliance or hemodynamics would have greatly helped this study. This limitation occurred because of time constraints: the need to return borrowed equipment and a shortage of mice. Fortunately, these problems can be addressed in follow-up experiments.

A third limitation of the data is the failure to localize OPN expression. This occurred due to technical problems with OPN immunostaining. Despite attempts with several different antibodies, the technical problem (localization of non-specific antibody to the same area) could not be overcome. Thus, though OPN expression was quantified before and after injury with western blot, the location of the expression is not known. In the laboratory, we are currently developing rabbit polyclonal anti-mouse antibodies that may help us address this problem. The presentation of altered adventitial collagen in the uninjured vessels of OPN null mice as compared to wild type suggests OPN is directly involved in organization of these collagen fibers. However, previous work in rats did not demonstrate the adventitia to be a site of OPN expression in uninjured vessels. It is possible the altered collagen appearance results from compliance effects on the movement of the vessel wall with each pulse wave. Possible solutions to this problem include the use of frozen sections instead of paraffin embedded sections, or the use of in-situ hybridization.

Despite these limitations, the data presented in this text does allow the projectís hypothesis to be addressed. **We accept the hypothesis. The data presented in this text clearly shows that the presence of osteopontin does affect the response to vascular injury.** However, the failure of the hypothesis to consider the expression of OPN prior to injury as a source of alteration of the injury response reveals the flawed reasoning in hypothesisí conception, and prevents achievement of the project goal (i.e. determination of the mechanism of action of OPN in vascular injury). As presented in the following sections, the continued advancement of this project centers on the formulation of a hypothesis that considers what was learned in the execution of this portion of the project.

Future plans

Though capable of addressing the projectís hypothesis, the data presented in this text does not adequately address the projectís goal, which was the determination of the mechanism by which OPN affects the vascular injury response. Given that OPN affects the function of uninjured blood vessels, the future goal requires an examination of the role of OPN in the function of uninjured vessels. Thus, though the overall goal remains the same, the future goal must be more specific. **The revised project goal is to mechanistically describe the role of osteopontin in normal vascular function.** The successful attainment of this goal will improve our understanding of the basic processes that underlie vascular function. As the data presented in this text identified OPN as a mediator of vascular compliance, the targeting of OPN may be therapeutically useful. As there are currently no drugs that directly alter vascular structure, if OPN proves to have direct action on the vasculature, then pharmacologic agents that act on OPN would represent a novel drug class. The revised project goal will be addressed with the **proposed hypothesis that osteopontin alters vascular function through a direct effect on collagen.** The projectís hypothesis could be tested with the following strategy.

Project strategy: Identify the effect of osteopontin on factors known to be involved with vascular function. To advance the project, two specific aims are proposed. Their successful achievement should allow the proposed hypothesis to be addressed, and provide substantial data toward the achievement of the revised project goal .

Specific Aim 1: Identify the structural basis for the difference in compliance between wild type and OPN null mice.

Specific Aim 2: Identify the basis for the difference in vascular tone between wild type and OPN null mice.

Research design

Specific Aim 1: Identify the structural basis for the difference in compliance between wild type and OPN null mice

Hypothesis: Osteopontin binding of collagen alters collagen fiber formation and organization.

Strategy: Comparison of electron micrographs of the vessel wall of OPN null mice to that of wild type mice in conjunction with osteopontin-collagen / elastin binding assays.

Is the altered compliance of OPN null mice accompanied by altered elastin/ collagen structure?

Regardless of whether the effect of OPN on the vasculature is primary or secondary to OPN action elsewhere, the arterial wall of OPN null mice is physically different than the equivalent arterial wall of wild type mice. This difference is not due to vascular tone as the pressure myograph experiment in chapter 3 was conducted in the presence of high concentration of vasodilator. Besides vascular tone, compliance is primarily mediated by collagen and elastin[13]. The lack of difference in the longitudinal extensibility of the carotid artery between groups suggests the difference in circumferential compliance to be due to altered collagen[18]. Additionally, adventitial collagen appeared less dense in OPN null mice as compared to wild type (Figure 16). Alterations of collagen with the loss of OPN is further supported by previous work as our laboratory[281] and others[283] have noted OPN null mice to have thicker collagen fibers in a less dense matrix than that of wild type mice.

As compliance is affected by the geometric arrangement of the collagen fibers as well as both the type and amounts of collagen expressed, analysis will include both quantification and examination of the collagen network. The carotid artery will be used as the composition of the aorta has been shown to vary along its length[20]. Carotid arteries will be harvested from groups of 16 week old wild type and OPN null mice (n=6 from each group), normalized for total protein, and then quantified for total collagen and elastin by western blot. Though collagen is normally insoluble, the quantification of total collagen by western blot has been described[288]. The quantification of collagen sub-type will be done by immunostaining. As collagen fibrillogenesis is known to be mediated by many factors and fibrils may form so as to mask the presence of a collagen sub-type, a brief pepsin digest will precede immunostaining. Our laboratory currently has serial cross-sections from carotid arteries of wild type and OPN null mice. These sections (n=6 from each group) will be immunostained with fluorescent tagged antibodies to collagens I, III, and V (Southern Biotech), and digitally photographed. Total light can then be used to quantify collagen sub-type in each vessel layer. Non-specific fluorescent labeled IgG will be used to assess non-specific binding. As it was successfully used in previous analyses of the OPN effect on collagen networks[281,283], examination of the collagen network will be done with a comparison of electron micrographs of cross sections of the carotid artery (n=6 from each group). The subjective work will be summarized with the selection of a representative section from each group.

The expected result of these experiments is to confirm the collagen structure of the arteries of OPN null mice to consist of thinner fibers in a less dense matrix than wild type. The work presented in the text (Figure 16) suggests elastin structure is not altered

in OPN null mice. Consistent with this result, there are no reports of OPN binding elastin. However, Giachelli et al. used immunostaining to localize OPN near the elastic laminae[274]. Thus, it would not be especially surprising to find the ultrastructure of elastin to be altered by the loss of OPN.

A problem with the electron microscope experiments is the lack of quantitation. The difference in elastin/ collagen structure between groups would have to be obvious for the experiments to be at all convincing. Though this is the expected result for collagen, if the result is not obvious, then the quantification of density of collagen matrices will be attempted using a variation of a procedure described by Ramanujan et al.[289] Briefly, fluourescein labeled dextrans of variable molecular weight (Sigma) will be allowed to permeate the freshly harvested vessels of wild type and OPN null mice. The tissue permeation should not be a problem as Netti et al. were able to monitor the flow of large molecules through tumor tissue[290]. Assuming success, the vessels successfully permeated with the heaviest dextran will then have a small (20 μm radius) spot exposed to 488 nm laser light so as to permanently bleach the exposed fluorochromes. The increase in light over time can then be measured as unbleached fluorochromes diffuse into the area to provide a measure of barrier to diffusion. Whereas the barrier to diffusion of small molecules is primarily effected by proteoglycans, the movement of large molecules is primarily effected by the collagen matrix[290]. Comparison of the diffusion times for large molecules should then provide a quantitative measure of density of the collagen network.

Does osteopontin colocalize with collagen or elastin?

Both Butler[186] and Kaartinen et al.[201] demonstrated OPN binding wells coated with collagens I-V. Butler reported dissociation constants ranging from 20-50 nM, and Kaartinen showed collagen type V binding to be stronger than type I and type I binding stronger than type III. However the in vivo binding of OPN to collagen has not been shown. Assuming the technical difficulties with immunostaining can be resolved, the OPN antibody will be tagged with a fluorochrome (Fluoreporter labeling kit, Molecular Probes), and used to demonstrate colocalization in confocal micrographs with the fluorescent tagged collagen antibodies (Southern Biotech) and with elastin antibodies (Elastin Products Company). With proper selection of fluorochromes, the excitiation of one fluorochrome by the light emitted from the other fluorochrome can be used to localize the antibodies to be close (10-100 angstroms) to each other. However, as background fluoresence levels need to be especially low for FRET to be valid, and staining ofextracellular matrix typically shows at least some background staining, a false positive result may occur. Thus, as always, good controls are necessary. The demonstration of fluoresence resonance energy transfer will clarify the colocalization as it will rule out all but very small bridge molecules. Even if fluoresence resonance energy transfer is not possible in this system, the experiment will verify or rule out colocalization.

The expected result of this experiment is the demonstration of colocalization with fluoresence resonance energy transfer of OPN with collagen in the medial layer. The binding of osteopontin to collagen has been described and the presence of both has been described in the medial layer. Though the work in this text showed collagen structure to be altered in the adventitial layer of OPN null mice, Giachelli et al. showed osteopontin not to be expressed in this layer[274]. The colocalization of osteopontin with elastin is also

expected as Giachelli et al. have already made this observation[274], but the observation of fluoresence resonance energy transfer with the elastin colocalization would be especially interesting as the binding of osteopontin to elastin has not been reported. The requirement for a bridge protein for the binding of OPN to elastin/ collagen will be tested using in vitro experiments which will be described.

Does increasing concentration of osteopontin further decrease circumferential compliance?

In chapter 3 a model of the action of OPN proposed OPN null mice to have greater vascular tone in compensation for their increased compliance. As chapter 1 describes that vascular compliance can be altered by hemodynamics, it is equally possible the increased compliance of OPN null mice occurs in compensation for increased vascular tone. To determine whether the action of OPN on the vasculature is direct or secondary to its action at another site, the compliance of vessels with an increased concentration of OPN will be measured. As osteopontin transgenic mice are available, the effect of increased OPN expression on compliance can be determined by comparison of pressure myograph measurements of compliance of these mice to those presented in chapter 3.

In addition, the effect of the increased expression of OPN following ligation will be determined by comparison of pressure myograph measures of compliance of the 4, 14, and 28 day ligated vessels of wild type mice to those of OPN null mice. As it is unlikely the local increase in OPN will affect any other sites in the body, a further decrease in circumferential compliance in response to increased vascular OPN would suggest a direct effect. As demonstrated in chapter 3, ligation of the left common carotid artery results in increased expression of OPN in the region around the ligature. Groups of wild type mice

(n=6) will have left common carotid arteries ligated, then harvested at 4 days, 2 weeks, 4 weeks, and 8 weeks. Compliance of the vessels will be determined using the pressure myograph technique also described in chapter 3. As they can also affect compliance, following measurement, collagen and elastin will be quantified by western blot and immunostain as described above.

Though not conclusive, these are especially exciting experiments as they will provide evidence as to the vessel being a primary site of osteopontin action. The data presented in this text showed less constrictive remodeling in wild type mice at a time of increased OPN expression. The further decrease of compliance in response to additional OPN could explain this result. Certainly there are other molecular changes induced by ligation (chapter one), but few follow the kinetics of OPN. Tracking changes in compliance with the change in OPN over time will either refute or further support a role for OPN in mediating compliance. The drawback of the experiment is it can not control for other differentially expressed proteins that follow OPN kinetics. Thus, the observed effect, if there is one, may be due to the action of a protein other than OPN, so the expected result of this experiment is evidence that further supports OPN mediation of the compliance of the carotid artery.

Does osteopontin have multiple collagen or elastin binding sites?

Though the use of recombinant OPN in the binding assays of Kaartinen et al. demonstrated post-translational modification of OPN is not necessary for collagen binding201, previous binding assays did not identify the specific collagen binding site(s) on OPN. The binding domains required for OPN binding of collagen will be determined by measuring the ability of increasingly smaller fragments of OPN to bind collagen.

Interestingly Kaartinen et al. found the binding of collagen by OPN to be calcium dependent[201], suggesting the need to retain the calcium binding sites of OPN (aa 86-95 and aa 216-228) for collagen binding. Our laboratory currently has recombinant expressed proteins equating to full-length OPN and fragments resulting from the matrix metalloproteinase cleavage of OPN (OPN40kD: aa 17-166, OPN32kD: aa 167-314, OPN25kD: aa 211-314, OPN15kD: aa 167-210). These fragments can be used to further localize the binding site(s). The OPN fragments/ domains will be quantified, radiolabeled (Custom label service, Amersham Bioscience) then serially diluted. The solutions will then be placed in wells coated with known amounts of collagens I-V. Wells coated with bovine serum albumin will be used as negative control[201]. The free OPN will then be washed from the wells, and bound OPN will be quantified using the radiation emitted from each well. Though the Scatchard plot has been used for determination of binding strength and valence, it has been criticized both in theory[291] and the way it has been applied[292]. A simpler and more accurate analysis has been described by Lodish et al.[293] From the data, a plot of bound OPN vs. OPN dilution will be made. Non-specific binding will be determined by carrying out the same experiment in the presence of 100 fold excess unlabeled OPN. As the specific binding sites will be occupied by excess OPN, labeled OPN will represent non-specific binding at each concentration. The specific binding at each concentration can then be determined by subtraction of non-specific binding from the total binding determined by the first experiment. The valence can be determined by the number of bumps in the curve, or, as it is possible different sites may have the same affinity, from the amount of OPN required to saturate a known amount of collagen / elastin. As the binding constant KD is defined as the concentration of ligand necessary to

bind half the receptors, inspection of the graph will provide a close approximation. The application of curvilinear regression to the data can locate KD with greater precision. Our recombinant protein expression system will then be used to express smaller OPN fragments to further refine the required protein region(s).

Elastin will also be assayed for its ability to bind OPN. Should such binding occur, the OPN binding sites for elastin will be similarly determined. The identification of multiple collagen or elastin binding sites would indicate OPN ability to form aggregates.

Can osteopontin alter the compliance of elastin/ collagen matrices *in vitro*?

In vitro work will also include analysis of the effect of OPN binding on the physical properties of elastin and collagen matrices. The formation of collagen[294] and elastin[295] matrix disks have been described. Separate disks containing collagen I-V and elastin will be made with increasing amounts of OPN (the upper limit to be 10x the concentration of OPN in injured vessels). Bovine serum albumin will be substituted for OPN as a negative control. The deformation of the disks in response to the application of force will then be quantified from digital photographs to provide measures of compliance. Additional experiments will be done with OPN fragments/ domains as indicated by the binding assays.

The drawback of this experiment is its inability to accurately represent the geometry of the matrix in vivo. Collagen fibrillogenesis is known to be mediated by many factors (e.g. temperature), and the complexity of in vivo regulation of collagen fibrillogenesis simply can not be replicated in vitro. However, the study of the effects of OPN fragments in this system could provide the mechanism by which OPN mediates compliance as well as the specific domains necessary. The expected result of this

experiment is the disks containing OPN (or the collagen binding domains) and collagen will demonstrate decreased compliance through a mechanism that involves OPN bridging.

Specific Aim 2: Identify the basis for the difference in vascular tone between wild type and OPN null mice.

Hypothesis: The presence of osteopontin decreases the reactivity of smooth muscle cells to vasoconstricting hormones.

Strategy: Comparison of amounts of factors known to affect vascular tone between wild type and OPN null mice in conjunction with reactivity assays.

Do OPN null mice show greater vascular tone?

Our model of the action of OPN in blood vessels has smooth muscle cells showing increased tone in the absence of OPN. The evidence supporting this aspect of the model is weak as we compared the in situ diameter of arteries of anesthetized mice to diameters predicted by arteries in vitro treated with vasodilator. A better comparison can be made with measurements of the exposed carotid arteries of anesthetized mice simultaneously monitored for blood pressure, blood flow and heart rate. The response of the arteries to vasodilators and vasoconstrictors can then be determined. It is possible that the manipulation of heart rate and blood pressure of anesthetized mice with epinephrine and intravenous fluids may allow the hemodynamics of alert mice to be replicated. Interestingly, atropine has little, if any, effect on mice due to their poorly developed parasympathetic nervous system,

Smooth muscle cell reactivity will be quantified with functional assays. Smooth muscle cells from OPN null mice will be grown on elastin and collagen matrix disks

described earlier in both the presence and absence of OPN. Similar to the previous experiments, the deformation of the disks in response to being released from the dish will then be quantified. The experiment will be repeated in the presence / absence of known vasoconstrictors / vasodilators. The resulting data will address the reactivity of smooth muscle cells to vasodilators / vasoconstrictors in the presence / absence of OPN.

It is expected that these experiments will identify a quantitative difference in either vasodilators/ vasoconstrictors, their associated receptors, or smooth muscle cell reactivity. To confirm the result in vivo, carotid arteries will be harvested from wild type (n=6) and OPN null mice (n=6), mounted in a pressure myograph. The vessels will then be inflated to 140 mm Hg, and digitally photographed before and after treatment with vasodilators/ vasoconstrictors identified in earlier experiments to be differentially involved. The response can be measured against the pressure diameter curves shown in chapter 3.

Though OPN is a substrate for smooth muscle cell attachment[285], it may be that OPN affects smooth muscle cell binding to collagen or elastin through competition for binding sites. If this were true, OPN null mice show greater vascular tone simply because they grip the extracellular matrix better. While this may appear unlikely, our laboratory has observed the presence of OPN and specific fragments of OPN to substantially affect cell adhesion (unpublished results). To rule out OPN effects on smooth muscle cell adhesion, the collagen and elastin disks in the presence of OPN and fragments of OPN will be plated with a known number of cells. The cells will be allowed to set on the disk for ten minutes, then unattached cells will be washed off. Comparison of the number of attached cells will demonstrate the effect of OPN on cell binding to extracellular matrix.

Limitations of the research design

The proposed research design thoroughly examines the interaction of OPN with collagen and elastin. Additionally, the experiments will identify the domains of OPN necessary for interaction with collagen and possibly with elastin. As OPN contains no known domains for these interactions, the domains identified will be novel and may contribute to our understanding of other proteins. Furthermore, the proposed experiments will identify the role of OPN in mediating vascular tone. Together these experiments will allow the hypothesis that OPN affects vascular function through a direct interaction with collagen to be addressed, and will provide critical information for the project goal of a mechanistic description of OPN function in normal vessels. As described above, if OPN does indeed have primary effects on the vasculature, these experiments could identify it as a target for a new class of drugs. For these reasons, the proposed experiments, though relatively simple, may be quite rewarding. However, the proposed design has two major limitations: 1) If OPN does not have direct action on the vasculature, then the proposed experiments do very little to identify the primary site of action. Should the proposed experiments show that OPN does not directly act on blood vessels, the differences in smooth muscle cell reactivity and vasoconstrictor/ vasodilator/ receptor levels in OPN null mice from that of wild type mice may provide insights to some candidate sites of OPN action. 2) The experiments will not address the original project goal of this text, determination of the role of osteopontin in vascular injury. Addressing the role of osteopontin in vascular injury using the original strategy will require OPN null mice that have been somehow normalized to wild type vascular function. The data that will result from these proposed experiments should provide insights into how such normalization could be done.

In general, the proposed experiments are relatively simple. As demonstrated by the work presented in this text, many of the procedures have already been practiced. Other potentially challenging tasks, such as recombinant protein expression, have been worked out by others in the laboratory. The greatest technical problem in carrying out the experiments is the lack of a key piece of equipment. The proposed research design relies heavily upon electron microscopy to demonstrate structural differences between wild type and OPN null mice. Unfortunately our laboratory possesses neither an electron microscope nor the sample preparation materials. Thus, this aspect of the project will be addressed through collaboration. One possible collaborator is the electron microscopy facility at the University of Maine.

Timeline for the proposed research design

The anticipated time to complete the proposed experiments is two years. A rough estimate of the sequence and time commitment to the different parts of the project are shown in Figure 23.

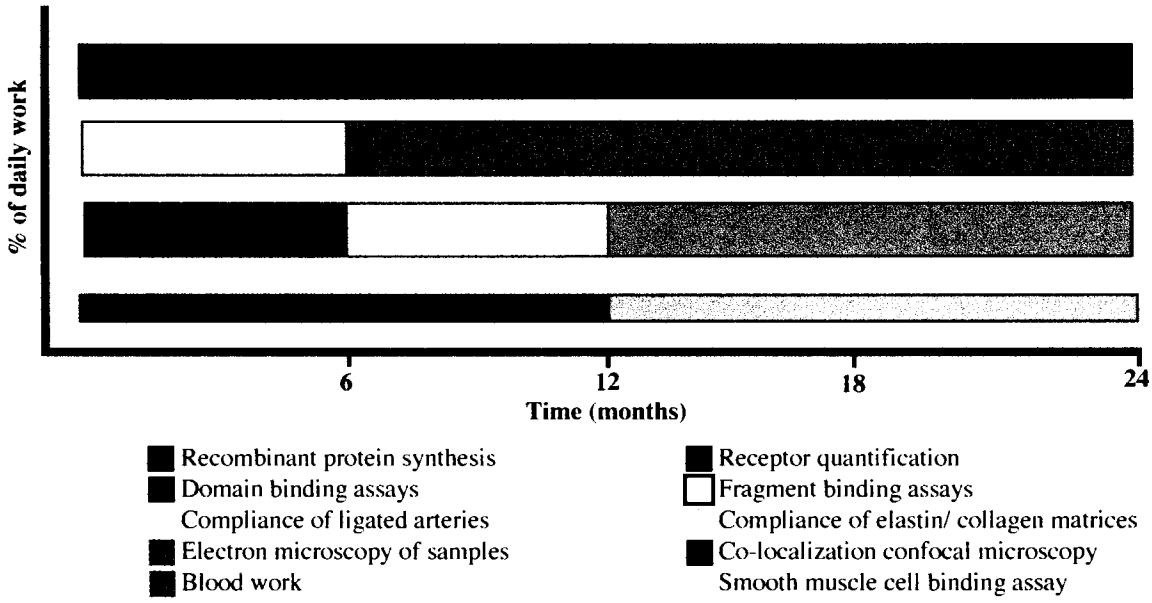


Figure 23. Anticipated sequence and time requirements for the different phases of the proposed project.

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