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OSTEOPONTIN STRUCTURE AND FUNCTION

by

Laura Lee Smith

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

Doctor of Philosophy

University of Washington

1998

Approved by _____
Chairperson of Supervisory Committee

Program Authorized to Offer Degree Pathology

Date 2/27/98

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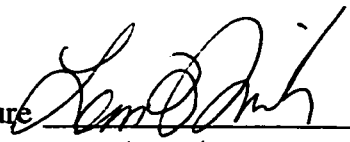
Signature 

Date 2/27/98

University of Washington

Abstract

OSTEOPONTIN STRUCTURE AND FUNCTION

by Laura Lee Smith

Chairperson of the Supervisory Committee:
Associate Research Professor Cecilia M. Giachelli

Department of Pathology

Osteopontin is an adhesive glycoprotein implicated in numerous diseases associated with inflammation and remodeling. There are several structural domains in osteopontin of particular interest. The RGD motif is a cell-attachment sequence shown to be critical for cell adhesion through α_v-containing integrins. In close proximity to the RGD domain is the thrombin cleavage site. Previous observations suggest that thrombin cleavage of osteopontin occurs *in vivo* and may be physiologically important. To study the functional significance of osteopontin cleavage by thrombin, we made osteopontin fusion proteins that contain either the N- or C-terminal domains expected to be formed following thrombin cleavage. We compared these fragments with native osteopontin in their ability to support adhesion of several cell lines, and identified the receptors mediating these interactions. Our data show that the N-terminal fragment supported adhesion of a melanoma cell line unable to bind native osteopontin, suggesting that osteopontin contains a cryptic binding activity. The receptor was identified as the $\alpha_9\beta_1$ integrin: a novel osteopontin receptor. In addition to adhesion, we show that $\alpha_9\beta_1$ can mediate cell migration, a function not previously identified for this integrin. To determine the domain important for $\alpha_9\beta_1$ interactions, we made mutations in the RGD region of the osteopontin fragment. Mutation of RGD to RAA, or eliminating the RGD completely, failed to support cell adhesion and migration, suggesting that the RGD domain was critical for this interaction. In contrast, $\alpha_9\beta_1$-mediated adhesion to tenascin was RGD-independent. These data demonstrate that $\alpha_9\beta_1$ is

one of the few integrin receptors that can interact with two distinct RGD-containing ligands through different adhesive domains.

CD44, a non-integrin, multifunctional adhesion molecule was recently identified as an osteopontin receptor. To analyze which forms of CD44 bind to osteopontin, we used a variety of CD44-immunoglobulin fusion proteins in enzyme-linked immunosorbant assays. Our data show that although the CD44-hIg fusion proteins could interact with hyaluronic acid as expected, there was no interaction between CD44H, CD44E, CD44v3,v8-v10, or CD44v3 with osteopontin. These studies suggest that CD44-osteopontin interactions may not be common *in vivo* and may be limited to a specific CD44 isoform(s), and/or a particular modified form of osteopontin.

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List of Abbreviations

BSA	bovine serum albumin
BAEC	bovine arotic endothelial cell
CD44-hIg	CD44-immunoglobulin
COL	collagen
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FN	fibronectin
GST	glutathione S-transferase
HA	hyaloronic acid
LN	laminin
mAb	monoclonal antibody
OPN	osteopontin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
RGD	arginine-glycine-aspartic acid
RGE	arginine-glycine-glutamic acid
RAA	arginine-alanine-alanine
SD	standard deviation
SDS	sodium dodecyl sulphate
SMC	smooth muscle cell
TN	tenascin
VEGF	vascular endothelial growth factor
10N	osteopontin N-terminal fragment
30N	osteopontin N-terminal fragment
10C	osteopontin C-terminal fragment

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For my husband, Rod Barnard

CHAPTER 1

INTRODUCTION AND BACKGROUND

An important component of the cellular environment is the extracellular matrix (ECM). The matrix is composed of locally secreted glycoproteins, proteoglycans and glycosaminoglycans. The ECM forms a network that not only provides bulk, shape, and strength of many tissues, but also influences the behavior of the cells within it. Glycosaminoglycan and proteoglycans molecules form a hydrated substance providing bulk and resistance to compressive forces. Fibrous protein such as collagen and elastin provide structure and strength. Other fibrous proteins such as fibronectin and laminin promotes cell attachment to the matrix. In addition to these functions, cell-matrix interactions regulates a number of activities including proliferation, migration, survival, and differentiation. The regulation involves sequestering and molecular rearrangement of the molecules in the ECM, attachment (or detachment) from the matrix, and integration of the ECM with the cellular cytoskeleton through specific surface receptors. Matricellular proteins are one of the important ECM components that participate in these regulatory events. "Matricellular proteins" are a family of non-structural extracellular adhesive molecules such as thrombospondin, SPARC, and tenascin (Bornstein, 1995). An important feature of this protein family is their ability to bind cell surface receptors as well as proteases, cytokines, and extracellular matrix proteins through their modular domains. Of particular interest, is the glycoprotein osteopontin, which is an adhesive molecule shown to be a critical component of several inflammatory and fibrotic diseases. Like other matricelluar proteins, most of osteopontin's roles are non-structural, however its localization to cement lines at the junction between older and newer bone suggests that this protein may also have a structural component in bone.

Osteopontin is a protein with diverse functions. It has been implicated in bone mineralization, ectopic calcification, tumor metastasis, inflammation and wound repair. This protein is also thought to be important in a number of vascular diseases including atherosclerosis and restenosis. Like other adhesive proteins, osteopontin is composed of multiple structural domains, which may be important for distinct functions. The arginine-glycine-aspartate (RGD) domain is an adhesive motif found in many matrix molecules, and is critical for α_V-mediated cell adhesion and migration to osteopontin. Osteopontin also contains a thrombin-cleavage site in close proximity to the RGD domain. Previous studies by others suggests that osteopontin is cleaved by thrombin *in vivo* and may have physiological importance. In addition, both osteopontin and thrombin are likely to be

localized together at sites of injury, inflammation, angiogenesis and in tumors. The long term goal of this project is to study the structure and function of osteopontin and the potential roles of osteopontin and its receptors in vascular diseases. Because thrombin-cleaved osteopontin peptides have been found *in vivo*, it now becomes important to determine if proteolytic fragmentation of osteopontin occurs in the vasculature and to determine if osteopontin fragments are functionally significant. The overall focus of this dissertation is based on two hypthoseses: 1) specific structural domains of osteopontin are critical for function and 2) proteolysis of osteopontin generates functional fragments and may be important for development of vascular diseases. The studies contained in this dissertation specifically address the regulation of osteopontin receptor specificity and function by proteolytic cleavage and the identification of functional domains in the proteolytically-cleaved osteopontin fragment. Chapter 1 provides an introduction and background information about osteopontin and the potential role of osteopontin in vascular diseases. The studies in chapter 2 address the biological significance of proteolytically cleaved osteopontin and identification of $\alpha_9\beta_1$ as a novel osteopontin receptor. Chapter 3 describes studies identifying the functional domain in the osteopontin fragment critical for $\alpha_9\beta_1$-mediated cell adhesion and migration, and compares the mechanism of receptor interaction with two $\alpha_9\beta_1$ ligands. Chapters 4 explores the interaction of osteopontin and its proteolytic fragments with the CD44 receptor. Conclusions for these studies and a proposal for future experiments are found in chapter 5.

Tissue Distribution of Osteopontin

Osteopontin is a secreted RGD-containing phosphoprotein of Mr~66kD. The tissue distribution and structure suggest it is a protein with diverse functions. In early studies, osteopontin was isolated from the bone (Fisher et al., 1987; Franzen and Heinegard, 1985; Gerstenfeld et al., 1990; Prince et al., 1987) In this tissue it is thought to be important in attachment of osteoclasts and bone resorption (Reinholt et al., 1990; Ross et al., 1993) Osteopontin is also present in urine, where it is an inhibitor of calcium oxalate crystal growth (Shiraga et al., 1992), and in some immune cells (Miyazaki et al., 1990; Patarca et al., 1989; Patarca et al., 1993), where it may play a role in immune resistance to certain bacteria and viruses (Patarca et al., 1993). In addition, osteopontin is found in cartilage (Mark et al., 1988; McKee et al., 1992), kidney (Giachelli et al., 1994; Nomura et al., 1988; Yoon et al., 1987), decidua and placenta (Mark et al., 1988; Nomura et al., 1988)

and a variety of biological fluids. Interestingly, osteopontin is also expressed in diseased tissues. High levels are observed surrounding carcinomas and sarcomas (Senger et al., 1988; Senger et al., 1989) and in various tissues undergoing dystrophic calcification, inflammation, remodeling and repair. For example, it is elevated in renal diseases (Giachelli et al., 1994; Pichler et al., 1994), atherosclerosis, myocardial necrosis, aortic valvular lesions and healing wounds. (Giachelli et al., 1995; Giachelli et al., 1997; Giachelli et al., 1995)). During development, osteopontin is expressed in the notochord (Thayer et al., 1995) and areas of ossification (Nomura et al., 1988). Importantly, osteopontin is also seen in the ductus arteriosus at a time when it remodels to form a neointima (Thayer et al., 1995). The multifunctional properties of osteopontin may be explained in part, by its multidomain structure discussed below.

Protein Structure

Osteopontin contains a number of domains thought to be critical for its function. The structure of osteopontin and some of its important features are shown in figure 1.1.

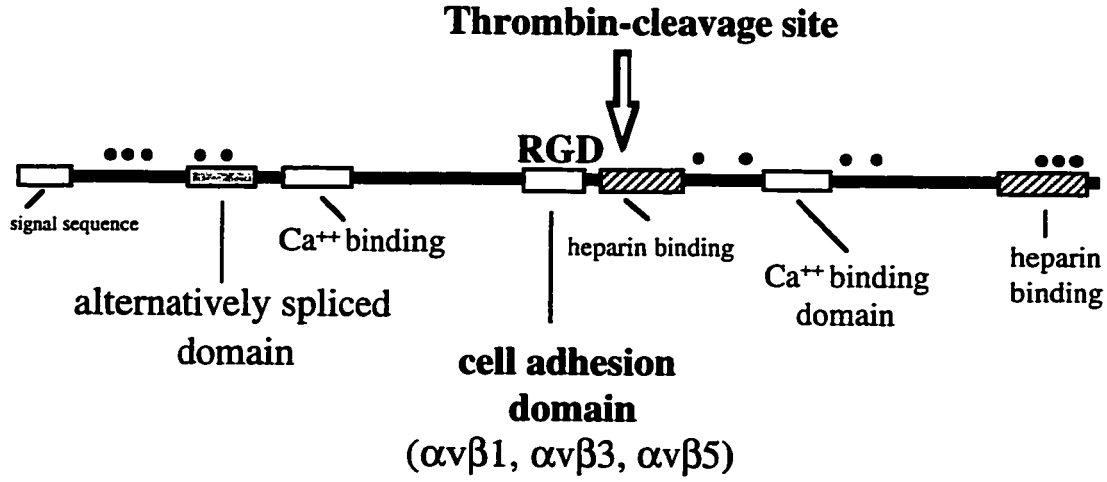

Figure 1.1. Structural map of osteopontin. Osteopontin is composed of multiple domains including an RGD adhesive sequence, several heparin binding homology domains and several highly acidic Ca^{++} binding regions. The thrombin cleavage site is 6 amino acids C-terminal from the RGD site. An alternatively spliced domain contains exon 5, which includes 14 additional amino acids.

The RGD domain is the cell attachment site of a large number of adhesive extracellular matrix proteins, (Hynes, 1992), and is critical for αv-integrin dependent cell adhesion and migration to osteopontin (Liaw et al., 1995; Xuan et al., 1995). In addition to binding integrin receptors, osteopontin can interact with other extracellular matrix molecules such as fibronectin and collagen (Chen et al., 1992; Nemir et al., 1989). Through these interactions, osteopontin may act as an adaptor protein, which bridges the cell surface with the surrounding matrix environment. Other conserved regions of osteopontin include a polyaspartic acid region, two heparin binding consensus sequences, an EF-hand-like calcium binding motif and numerous phosphorylation and glycosylation sites (Prince, 1989). Although native osteopontin is often phosphorylated and glycosylated, these post-translational modifications do not always effect its function *in vitro*. Osteopontin is also susceptible to proteolytic fragmentation. There are two conserved thrombin cleavage sites in human osteopontin. The major thrombin-cleavage site is 6 amino acids C-terminal from the RGD domain at the Arg^{169}- Ser^{170} residues. A second potential thrombin-cleavage site is within the RGD domain (Arg^{160}-Gly^{161}) (Senger et al., 1989). Osteopontin is also susceptible to trypsin (Zhang et al., 1990) and endoproteinase Arg-C (van Dijk et al., 1993). The significance of the thrombin-cleavage site is a focus of this dissertation and will be discussed further in the introduction.

Osteopontin Gene Structure

The human osteopontin gene is 9 kb and maps to 4q13 in the human genome (Young et al., 1990). In the mouse, the osteopontin gene maps to the ric locus on chromosome 5 which determines susceptibility to infection by *Rickettsia tsutsugamushi*. (Fet et al., 1989; Patarca et al., 1989). Interestingly, Patarca et al., correlated specific osteopontin alleles with *Rickettsia* resistance. The Opn^a allele is associated with resistance whereas the Opn^b and Opn^c mouse strains die from widespread infection after intraperitoneal inoculation of this bacteria. Although most of the functional regions of the osteopontin protein are the same in the different alleles, the proteins differ in at least 10 amino acids including several substitutions in the calcium binding domain and the heparin binding homology region (Ono et al., 1995).

There are three splice variants of the osteopontin gene. Two of these varants are due to variable usage of exon 5 which contains 14 amino acids, including several potential phosphorylation and glycosylation sites (Young et al., 1990). The third variant is a cell-type specific splicing of exon 1 which results in a 52-bp insertion in a non-coding region (Singh et al., 1992). The functional significance of osteopontin splice variants is not yet known.

Osteopontin Receptors

Cellular interactions with osteopontin thus far described are mediated through integrin receptors. Integrins are a family of heterodimeric glycoproteins consisting of distinct α and β subunits. At least 22 different heterodimeric (αβ) dimers have been reported so far. The different combinations of α and β heterodimers that form functional receptors are shown in figure 1.2.

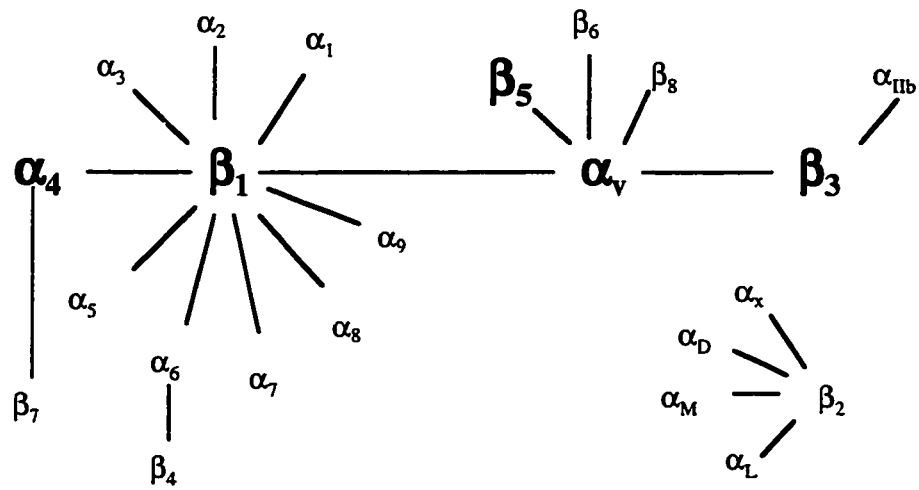

Figure 1.2. Intergrin receptor subunits. The α and β subunits heterodimerize to form ingegrin receptors. The known osteopontin receptors are shown in bold.

The integrins are receptors that mediate cell-cell and cell-extracellular matrix interactions. Many of the adhesive proteins contain an RGD sequence, which is the cell attachment site

from nearly half of the known integrins. Interaction with the matrix serves to link matrix molecules to the actin cytoskeleton, resulting in cytoskeletal reorganization and initiation of signal transduction cascades. Through this process, integrins can regulate cell differentiation, proliferation and survival, and induce cell adhesion and migration. In addition, integrins can physically modify the extracellular microenvironment by transmitting contractile forces or by inducing the cells to secrete matrix-degrading proteases (Hynes, 1992; Meredith et al., 1993). Although the signaling cascades are still being investigated, it is becoming clear that numerous cytoplasmic proteins are co-localized with integrins in focal adhesion sites and are involved in the signaling processes.

In addition to cytosketal rearrangement and signaling cascades that are initiated by ligand binding, integrins can also mediate adhesion through an activation process. Integrin activation alters the conformation of the receptor and increases its affinity for ligand. Although this process is not well understood, factors from outside the cell have been shown to regulate integrin affinity. These include agents such as Mn^{++}, certain activation monoclonal antibodies, and ligand peptides (S'Anchez et al., 1996). Affinity modulation can also be controlled from the cell interior. For example, activation of integrin $\alpha_{IIb}\beta_3$ by thrombin is initiated by signalling through the thrombin receptor, and increases the affinity of $\alpha_{IIb}\beta_3$ for fibrinogen and leads to platelet aggregation (Phillips et al., 1991).

Osteopontin is a ligand for a number of integrin receptors including $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, and $\alpha_4\beta_1$. The $\alpha_v\beta_3$ receptor is particularly interesting. This integrin allows a variety of cell types to attach and migrate to osteopontin (D'Errico et al., 1995; Helfrich et al., 1992; Liaw et al., 1994; Liaw et al., 1995; Liaw et al., 1995), including smooth muscle cells and endothelial cells. Additionally, $\alpha_v\beta_3$ provides survival signals for endothelial cells adherent to osteopontin, suggesting that $\alpha_v\beta_3$-osteopontin interactions may inhibit apoptosis (Scatena, 1997). The integrins $\alpha_v\beta_5$ and $\alpha_v\beta_1$ were also found to mediate adhesion of human aortic smooth muscle cells (SMC) (Liaw et al., 1995) and an interaction between $\alpha_4\beta_1$ and osteopontin was recently reported using a macrophage line, P388D1 (Nasu et al., 1995). The ligation of osteopontin with the different receptors has distinct consequences. For example, although $\alpha_v\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_3$ all bind through the RGD domain and can mediate adhesion, $\alpha_v\beta_3$ is the only known receptor to induce cell migration to osteopontin. (Liaw et al., 1995). More recently, Weber et al, identified osteopontin as a ligand for

CD44, a non-integrin, cell surface glycoprotein (Weber et al., 1996). In that report, osteopontin could mediate cell adhesion and migration through the CD44 receptor. The potential significance this interaction is discussed below.

CD44-Osteopontin interactions

CD44 is a multifunctional proteoglycan. It participates in diverse cellular functions such lymphocyte activation, recirculation and homing, tumor metastasis, and hematopoiesis (Haynes et al., 1989; Lesley et al., 1993; Underhill, 1992). The extracellular matrix ligands of CD44 include hyaluronate (Aruffo et al., 1990; Underhill, 1992), fibronectin (Jalkanen and Jalkanen, 1992), and collagen (Carter and Wayner, 1988; Gallatin et al., 1989). Several different isoforms of CD44 exist. These isoforms are generated by alternative splicing of the RNA, and vary between 85 and 230 kD. In addition to alternative splicing, differential glycosylation can also lead to variants containing chondroitin sulphate and heparin sulfate (Cooper and Dougherty, 1995; Freed et al., 1989; Jackson et al., 1995; Labarriere et al., 1994). The functional significance of these unique isoforms is beginning to be appreciated. For example, although all CD44 variants contain the hyaluronate binding domain, their affinity for hyaluronate is variable (Stamenkovic et al., 1991; Sy et al., 1991) and depends on the glycosylation state of the CD44 molecule. Additionally, CD44 isoforms containing exon V3 are the only variants involved in presenting heparin sulfate-binding growth factors (Bennett et al., 1995). Many CD44 variant forms have also been associated with different types of tumor growth and metastasis. In recent studies by Weber et al (Weber et al., 1997; Weber et al., 1996), osteopontin was shown to be a ligand for CD44. These results are potentially very exciting because CD44 and osteopontin are often found associated together as sites of inflammation, tissue injury and tumor metastasis. Several studies also suggest that both molecules are expressed in remodeling vascular tissue (Jain et al., 1996), in kidney (Sibalic et al., 1997) and in angiogenic vessels (Griffioen et al., 1997). Additionally, CD44 and osteopontin both mediate adhesion and migration of smooth muscle cells: two processes important in vascular remodeling. Together these data implicate osteopontin-CD44 interactions in vascular diseases such as atherosclerosis and restenosis. However, the potential interaction between CD44 and osteopontin in vascular cells has not yet been investigated. Interestingly, the interaction site for CD44 on osteopontin was shown to be within the C-terminal half of the osteopontin molecule (Weber et al., 1997), making that report the first

to show a function for the C-terminal end of the osteopontin protein. Part of the studies in this dissertation were designed to investigate CD44-osteopontin binding and to determine if the heparin binding domain of the C-terminal fragment was responsible for its interaction.

Osteopontin Function in Vascular Disease

Smooth muscle cell proliferation and migration are important features in vascular diseases and thought to contribute to atherosclerosis and restenosis. Smooth muscle cells migrate form the tunica media to the intima, where they proliferate and form an intimal mass. Subsequent formation of a fibrous plaque leads to the advanced atherosclerotic lesion. In this process, the vascular smooth muscle cells are also likely to play an important role in determining the composition of the surrounding environment by secreting a variety of matrix proteins and matrix degrading enzymes. How these extracellular matrix components play a role in mediating the migratory, prolfierative, and survival properties of vascular cells is of considerable interest.

A number of studies have led to the hypothesis that osteopontin and its receptors contributes to the progression of vascular disease (Gailit and Ruoslahti, 1988; Giachelli et al., 1993; Giachelli et al., 1995). Osteopontin may mediate several events associated with atherosclerotic lesions including calcification, smooth muscle cell migration and angiogenesis. Smooth muscle cell migration is thought to be a critical process in lesion development and vascular wound repair. Both *in vitro* and *in vivo* data suggests that osteopontin may play a role in this process. *In vitro* studies demonstrate that osteopontin promotes adhesion and stimulates migration of smooth muscle cells (Liaw et al., 1994; Liaw et al., 1995). Recent studies by Weintraub et al also suggest that osteopontin can act as an autocrine factor to initiate smooth muscle cell invasion into extracellular matrices (Weintraub et al., 1996). *In vivo*, osteopontin is induced after balloon injury at a time coinciding with smooth muscle cell migration (Giachelli et al., 1993) but is not expressed in the vessel wall of the uninjured adult rat. The functional significance of osteopontin *in vivo* was shown by the ability of anti-osteopontin antibodies to reduce neointima formation following balloon injury. The data suggests that the 33% decrease in intima formation in animals receiving anti-osteopontin antibodies, may be due to decrease smooth muscle cell migration, providing additional support for this hypothesis (Liaw et al., 1997). Neointima formation was also reduced in the presence of antagonists to $\alpha_v\beta_3$ integrin (Choi et al.,

1994), the receptor that mediates osteopontin induced migration of smooth muscle cells (Liaw et al., 1995). In addition, several growth factors that are known to contribute to neointima formation, also induce osteopontin expression. For example, TGFβ, basic FGF (bFGF), and angiotensin II increased osteopontin mRNA and protein levels in smooth muscle cell cultures (Giachelli et al., 1993). Both osteopontin and $\alpha_v\beta_3$ were also upregulated during regeneration of wounded endothelium following balloon injury. This suggests a role for osteopontin and its receptor in the migration of endothelial cells during the restoration process of covering the injured lesion (Liaw et al., 1995).

In humans, osteopontin synthesis is absent in the normal vessel but is abundant in atherosclerotic plaque and restenotic lesions. Expression of osteopontin in the plaque was associated with the highly calcified areas, with the smooth muscle cells and infiltrating macrophages, and the endothelial cells in angiogenic vessels (Giachelli et al., 1993). The expression of osteopontin in areas of calcium deposits of atherosclerotic plaque, suggests a potential role in arterial calcification. Osteopontin was also highly expressed in the angiogenic vessels of the plaque. This was particularly interesting since one of its receptors, $\alpha_v\beta_3$, was also localized to this area (Hoshiga et al., 1995). This integrin was found to be required for angiogenesis in the chicken chorioallantoic membrane model (Brooks et al., 1994), and neovascularization of tumors *in vivo* (Brooks, 1994, cell 79:). The co-localization of osteopontin and $\alpha_v\beta_3$ in angiogenic vessels suggest osteopontin could be a key ligand for $\alpha_v\beta_3$-mediated angiogenesis. Figure 1.3 shows some of the processes in vascular remodeling where osteopontin is hypothesized to be important.

Protease-Extracellular Matrix Interactions

In addition to adhesive proteins such as osteopontin, proteolytic enzymes, including matrix metalloproteinases and members of the coagulation and fibrinolytic cascades, are often present at sites of inflammation and tissue remodeling. These proteases are thought to mediate many aspects of the extracellular matrix remodeling process (Birkedal, 1995; Chen, 1992). For example, by degrading specific matrix components and allowing cell detachment, matrix metalloproteinases are thought to be critical for smooth muscle cell migration in rat carotid arteries after experimental vascular injury (Bendeck et al., 1994; Zempo et al., 1994) and endothelial cell migration during angiogenesis (Mignatti and Rifkin, 1996). In addition, elevated levels of matrix metalloproteinases, are expressed at

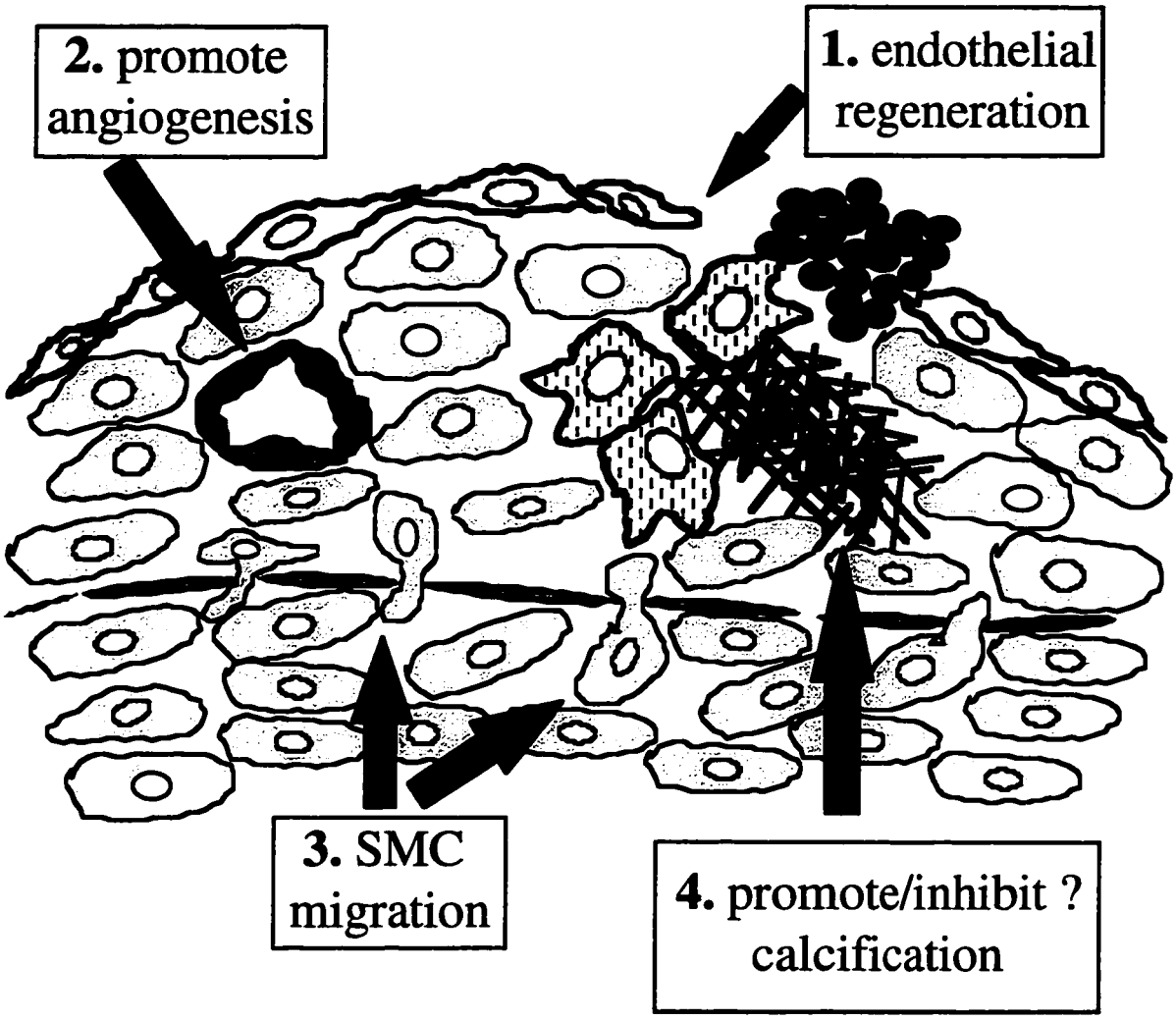

Figure 1.3. Hypothetical model for osteopontin's roles in vascular remodeling. Osteopontin may play a role in vascular remodeling by 1) stimulating migration and survival of edothelial cells as they cover an injured area and 2) promoting angiogenesis. In addition, osteopontin may 3) stimulate the migration of SMCs from the media into the intima, and 4) promote or inhibit vascular calcification.

sites of unstable atherosclerotic lesions (Galis et al., 1994; Nikkari et al., 1995). Degradation of the extra cellular matrix at these sites by matrix metalloproteinases may eventually lead to plaque rupture.

Proteases generated during coagulation may also play a role in remodeling. Plaque rupture, and vascular interventions cause platelet adhesion to the injured wall and activate the coagulation cascade and thrombin. Therefore in addition to matrix metalloproteinases, local concentrations of thrombin may be quite high in diseased vascular tissue. I have proposed that thrombin may also play a role in cell-matrix interaction by degrading matrix proteins that are susceptible to thrombin cleavage, such as osteopontin. Since osteopontin and thrombin are likely to be localized in tissues undergoing remodeling, it would not be surprising to find thrombin-cleaved osteopontin fragments generated at these sites. In fact, previous studies have shown that osteopontin proteolytic fragments are found at several sites *in vivo*. Senger *et al* demonstrated that rat osteopontin is cleaved during whole blood coagulation (Senger et al., 1989). Osteopontin purified from human milk also contains both the native osteopontin and the thrombin cleaved 35kD fragment (Senger et al., 1989). In addition, an intradermal injection of vascular endothelial growth factor (VEGF) and osteopontin resulted in rapid osteopontin fragmentation by endogenous thrombin, suggesting that the vascular permeability activity of VEGF may mediate thrombin cleavage of osteopontin *in vivo*.

Cellular Interactions During Tissue Remodeling

The matrix undergoes dynamic changes in composition and structure during tissue remodeling, due to the biosynthesis and proteolytic degradation of proteins. These changes must be continuously interpreted by the cell surface receptors such that appropriate changes in cell response can occur. Rapid cellular responses following protein degradation may come about by exposing cryptic activities following proteolytic degradation or by altering receptor specificity. For example, a 120 kD fragment of fibronectin induces the production of matrix metalloproteinase, which is a function not seen in the full length molecule (Werb et al., 1989). In addition, the conversion of fibrillar collagens into protelyzed forms correlates with changes in smooth muscle cell function (Koyama et al., 1996). It would not be surprising if thrombin altered the receptor specificity of osteopontin, or unveiled new osteopontin functions because a common feature of this protease is to reveal cryptic

activities. For example, thrombin cleavage of fibrinogen releases peptides that spontaneously form fibrin (Stubbs and Bode, 1995). Thrombin also cleaves its own receptor and the cleavage reveals a new NH_3 terminus that stimulates receptor activation (Brass et al., 1994).

Significance of osteopontin proteolytic fragmentation

It is clear that there is a delicate balance of biosynthetic and degradative pathways of the extracellular matrix in remodeling tissues. Tipping the balance one way or the other may accelerate the repair process or promote progression to a pathogenic state. An understanding of how proteases regulate osteopontin interactions with the cell will be important in recognizing the role osteopontin plays in remodeling tissues and vascular pathologies. In addition, identification of functional osteopontin domains and their surface receptors will aid in the development of new approaches to control osteopontin-associated pathologies.

The studies in this dissertation have been based on the hypthothesises that cleavage of osteopontin by thrombin generates functional fragments that might be important in vascular disease and that specific structural domains in osteopontin are critical for its function. To test these hypotheses, I generated recombinant fragments that would be formed following thrombin cleavage and tested their ability to support cell adhesion and migration in an *in vitro* system. Having found that the N-terminal domain of osteopontin was biologically functional, the receptors and the structural domain responsible for this interaction were then identified. Our data show that the N-terminal osteopontin fragment, which contains the RGD domain, supports adhesion through the $\alpha_v\beta_3$ integrin. Unexpectedly, we also found that this domain supports adhesion and migration of a cell line unable to bind native osteopontin. This suggests that osteopontin adhesive interactions may be regulated by thrombin cleavage. We also demonstrate hat osteopontin contains a cryptic binding activity which can be recognized by a novel osteopontin receptor. This receptor has been identified as the $\alpha_9\beta_1$ integrin. In addition, we found that the RGD domain was critical for these interactions. Reagents are now being generated that should specifically detect osteopontin cleaved fragments by immunocytochemistry *in vivo*.

CHAPTER 2

OSTEOPONTIN N-TERMINAL DOMAIN CONTAINS A CRYPTIC ADHESIVE SEQUENCE RECOGNIZED BY $\alpha_9\beta_1$ INTEGRIN

This work in similar form was published by Laura L. Smith, Hung-Kam Cheung, Leona E. Ling, John Chen, Dean Sheppard, Robert Pytela, and Cecilia M. Giachelli (*The Journal of Biological Chemistry*, 1996. Vol. 271, No. 45, pp. 28485-28491)

INTRODUCTION TO CHAPTER 2

Osteopontin is a multifunctional glycoprotein which promotes cell adhesion and migration. Previous studies have suggested that osteopontin plays a role in bone resorption, tumorigenesis and metastasis (Denhardt and Guo, 1993). More recently, osteopontin has been implicated in a number of disease states associated with inflammation and tissue remodeling (Giachelli et al., 1993; Giachelli et al., 1995; Giachelli et al., 1995; Murry et al., 1994; O'Brien et al., 1995).

Many cellular interactions with osteopontin are mediated through integrin receptors. Integrins are capable of generating signals that control many aspects of cell behavior including differentiation, adhesion, migration and apoptosis (Hynes, 1992; Meredith et al., 1993). The $\alpha_v\beta_3$ integrin allows a variety of cell types to adhere and migrate to osteopontin (D'Errico et al., 1995; Helfrich et al., 1992; Liaw et al., 1994; Liaw et al., 1995; Liaw et al., 1995). In addition to $\alpha_v\beta_3$, the $\alpha_v\beta_5$ and $\alpha_v\beta_1$ integrins were recently found to mediate adhesion of human aortic smooth muscle cells (SMC) (Liaw et al., 1995), and human embryonic kidney cells to osteopontin (Hu et al., 1995). A weak interaction was also demonstrated between $\alpha_4\beta_1$ and osteopontin in the macrophage line, P388D1 (Nasu et al., 1995). Interestingly, occupancy of osteopontin with different receptors has distinct functional consequences. For example, in SMCs, $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ mediate adhesion, but only $\alpha_v\beta_3$ can support migration (Liaw et al., 1995).

Osteopontin contains several interesting structural domains. The RGD domain is an adhesive motif found in many matrix molecules (Hynes, 1992), and is critical for α_v-integrin dependent cell adhesion and migration to osteopontin (Liaw et al., 1995; Xuan et al., 1995). Osteopontin is also susceptible to proteolytic fragmentation. There are two conserved thrombin cleavage sites in human osteopontin. The major thrombin-cleavage site is at residues Arg^{169}-Ser^{170} which is 6 amino acids C-terminal from the RGD domain. A second potential thrombin-cleavage site is within the RGD domain (Arg^{160}-Gly^{161}) (Senger et al., 1989). Previous studies have shown that osteopontin proteolytic fragments are found *in vivo* and may have physiological importance (Senger et al., 1989; Senger et

al., 1989). In addition, both osteopontin and thrombin are likely to be localized together at sites of injury, inflammation, angiogenesis and in tumors. The functional activity of cleaved osteopontin, however, is unclear. One report demonstrated that thrombin cleavage destroyed RGD-mediated cell adhesion (Xuan et al., 1994). In contrast, a second report showed that thrombin treatment enhanced osteopontin cell adhesive activity (Senger et al., 1994). One explanation for the discrepancy is that the interaction with osteopontin fragments may be mediated through distinct receptors in different cell types. A key to understanding the function of osteopontin, therefore, is identifying the receptors that interact with not only the full-length molecule, but any functional proteolytic fragments.

To study the functional significance of osteopontin fragmentation, we performed adhesion experiments using glutathione-S-transferase (GST)-osteopontin fusion proteins. For these studies, we created osteopontin peptides which contain either the N- or C-terminal domains expected to be formed following thrombin cleavage at the Arg^{169}-Ser^{170} site, which is 6 amino acids C-terminal from the RGD adhesive motif. We compared the osteopontin fragments in their ability to support adhesion of several different cell lines with that of native osteopontin and identified the receptors mediating these interactions. These studies show that the N-terminal fragment of osteopontin contains a functional RGD domain recognized by $\alpha_v\beta_3$, as well as a cryptic adhesive sequence recognized by the $\alpha_9\beta_1$ integrin.

MATERIALS AND METHODS

Cell lines - Bovine aortic endothelial cells were isolated from bovine aortas as previously described (Gajdusek and Schwartz, 1983). Mo and Moα_v melanoma cells were maintained in DMEM (GIBCO BRL) containing 10% fetal calf serum. Mo and Moα_v were both derived from M21 melanoma cells. One subclone, Moα_v expresses high levels of $\alpha_v\beta_3$. Mo is a subclone expressing no detectable levels of the α_v subunit. These cell lines were provided to us by Dr. Mark H. Ginsberg (The Scripps Research Institute) and have previously been described (Chen et al., 1995). Cell lines SW480 and 293 were obtained from ATCC.

Antibodies and Peptides - Monoclonal antibodies (mAb) against human β_1 (P4C10) (Carter et al., 1990), α_2 (P1E6) (Carter et al., 1990), α_3 (P1B5) (Wayner and Carter, 1987), α_5 (P1D6) (Wayner et al., 1988), and $\alpha_v\beta_5$ (P1F6) (Weinacker et al., 1994) integrins as well as GRGDSP and GRGESP peptides were purchased from GIBCO BRL. Monoclonal antibodies against human α_v (mAb 1980) (de Vries et al., 1986) , α_4 (P4G9) (Wayner et al., 1989), β_1 (LM534) (Giancotti and Ruoslahti, 1990) and $\alpha_v\beta_3$ (LM609) (Cheresh and Spiro, 1987) were purchased from Chemicon International Inc. Monoclonal antibody against human α_1 (5E8D9) (Luque et al., 1994) was purchased from Upstate Biotechnology Inc. Lake Placid, New York. The anti-β_3 antibody (SZ.21), was purchased from Immunotech, Westbrook, ME. Anti-α_v (L230) has been shown to block the function of all α_v integrins (Weinacker et al., 1994). The anti-α_v (LM142) (Cheresh and Spiro, 1987), was provided by Dr. Cheresh. Anti-$\alpha_{IIb}\beta_3$ antibody (D57) (Chen et al., 1994), was provided by Dr. Mark Ginsburg. Anti-α_6 (JIB5) is a mouse monoclonal antibody which has been previously characterized and was provided by Dr. Caroline Damsky (Damsky et al., 1992). Anti-α_9 antibody (1057) (Palmer et al., 1993) and anti-α_8 antibody (19946) (Schnapp et al., 1995), are both affinity purified polyclonal antibodies against the cytoplasmic domain of the corresponding integrin. The neutralizing α_9 monoclonal antibody (Y9A2) (Wang et al., 1995) and the anti-β_6 antibody (E7P6) (Weinacker et al., 1994) have been previously characterized.

Adhesive Proteins and Recombinant Osteopontin Fragments - Native osteopontin was purified from conditioned medium of rat pup smooth muscle cell cultures as previously described (Liaw et al., 1994). Laminin and fibronectin were purchased from GIBCO BRL (Gaithersburg, MD) and collagen I was purchased from Collaborative Biochemical Products (Bedford, MA).

Osteopontin N- and C-terminal proteins were generated by thrombin cleavage of bacterially expressed glutathione S-transferase-osteopontin (GST-OPN) fusion proteins. Expression plasmids containing GST-OPN were generated by cloning PCR amplified N- and C-terminal osteopontin fragments into Bam-H1/Eco-R1 sites of pGEX-2T (Pharmacia, Piscataway, NJ). The 5' primer CGCGGATCCATACCAGTTAAACAGGCT and the 3' primer TCCCCCGGGTCACCTCAGTCCATAAAC were used to amplify the N-terminal

osteopontin fragments, 10N and 30N from the plasmids OP10 and OP30 respectively. The plasmid OP10 (Young et al., 1990) was provided by Dr. Larry Fisher. OP30 (Kiefer et al., 1989) was obtained from ATCC. The C-terminal, 10C fragment, was amplified from OP10 using 5' primer CGCGGATCCAAATCTAAGAAGTTTCGC and 3' primer TCCCCCGGGTTAATTGACCTCAGAAGA. The GST-OPN fusion constructs were DNA sequence verified. *E. coli* JM109 cells transformed with these GST-OPN plasmids were grown in LB + 150 μg/ml ampicillin, then induced with 0.1 mM IPTG for 2 hours at 37° C to express the fusion proteins. The GST-OPN fusion proteins were purified basically according to the manufacturer's instuctions (GST gene fusion system, Pharmacia, Piscataway, NJ) with glutathione Sepharose beads. The OPN N- or C-terminal fragments was separated from GST-bound beads by treating with 0.1 units biotinylated-thrombin/μg GST-OPN (Novagen, Madison, WI) for two hours (either at room temperature for the 10C fragment or at 37°C for the 10N and 30N OPN fragments). The cleavage reaction was stopped with biotinlyated-PPACK (400 ng/unit biotinylated-thrombin). Supernatants were collected and biotinylated-thrombin and PPACK were removed by incubating with strepAvidin-agarose beads (Pierce, Rockford, IL) and separation of beads from supernatant.

The full-length recombinant osteopontin was generated as a histidine-tagged protein. An expression plasmid containing histidine-tagged osteopontin (his-OPN) was generated by cloning a PCR fragment containing the full-length splice variant of human osteopontin (OP10, (Young et al., 1990)), into the BamH1 site of vector pQE30 (Qiagen, Chatsworth CA). *E. coli* transformed with the his-OPN plasmid was grown in LB + 100 μg/ml ampicillin and induced with IPTG at 37°C to express the histidine-tagged protein. The his-OPN was purified from bacterial cells according to manufacturer's instructions (QIAexpressionist kit, Qiagen, Chatsworth CA), chromatographed on Ni-NTA resin, and eluted with 0.2 M imidazole. The purified his-OPN was analyzed by SDS-PAGE.

Cell Adhesion Assay - Adhesion of BAEC and melanoma cells to ligand coated microtiter plates was performed as described (Liaw et al., 1994). Briefly, matrix proteins or osteopontin fragments were coated onto 96 well maxisorp microtiter plates (Nunc Inc., Naperville, IL) overnight at 4°C, and then blocked 10 mg/ml bovine serum albumin (BSA) in PBS for 1 hour at 37°C. Cells were suspended in DMEM (melanoma cells) or

Waymouth's medium (BAEC) containing 1 mg/ml BSA, and preincubated with and without antibodies or peptides for 15 minutes at 37°C. Melanoma cells (100,000) or BAEC (30,000) were added to wells and allowed to incubate at 37°C for 1 hour. Absorbance (595 nM) of toluidine blue stained adherent cells was measured. Under these conditions, absorbance was proportional to cell number (Liaw et al., 1994). Cell adhesion assays for SW480 cells were performed as above with slight modifications (Busk et al., 1992).

FACS Analysis - Integrin expression was analyzed by fluorescent flow cytometry. After dispersion, 0.5 X 10^6 cells were treated with the primary antibody or an irrelevant antibody for 30 minutes at 4°C in binding buffer (PBS containing 2 mg/ml BSA and 0.02% NaN_3). They were then washed with the same buffer and incubated with secondary antibody conjugated to phycoerythrin (Biomeda, Foster City, CA) for 30 minutes at 4°C, washed twice with PBS and resuspended in PBS containing 2% paraformaldeyde for FACScan analysis (Becton Dickinson, Rutherford, NJ).

Cell Surface Biotinylation and Immunoprecipitation - Cell surface proteins were labeled with biotin essentially as described (Busch et al., 1989). A 5.0 X 10^6 suspension of cells was incubated with 1 mg/ml NHS-LC-biotin (Pierce, Rockford, IL) for 30 minutes on ice. Cells were washed three times with PBS and incubated in lysate buffer (PBS with 1% triton-X, 200 μM PMSF, 0.5 μg/ml leupeptin and 2 μg/ml aprotinin) at 4°C for 30 minutes. To immunoprecipitate surface biotinylated proteins, the lysate supernatant (2.0 X 10^6 cell equivalence) was added to PBS containing 1% triton, 0.5 mg/ml BSA and fresh protease inhibitors, and precleared with 40 μl of 50% (vol/vol) protein A-sepharose CL-4B (Pharmacia). The supernatants were immunoprecipitated with the anti-integrin antibody or a mouse IgG as a negative control, at 4°C. Immune complexes were recovered by binding to protein A-sepharose and washing five times with IP wash buffer (50 mmol/L Tris pH 7.4, 0.5 mol/L NaCl, 2 mmol/L PMSF, 0.1% Triton X-100, and 0.1 % Tween 20). After samples were separated by electrophoresis on 8% polyacrylamide-SDS gels under nonreducing condition, the proteins were transferred to polyvinylidene difluoride membrane (DuPont NEN). The membrane was blocked with 10% nonfat dry milk in TBST buffer (10 mmol/L Tris base pH 8, 150 mmol/L NaCl, and 0.05% Tween 20) at room temperature for 1 hour. After washing, blots were incubated for an additional hour with streptavidin-biotinylated horseradish peroxidase complex (Amersham, Arlington

Heights, IL), and proteins were visualized by the addition of a chemiluminescence reagent according to the manufacturer's instructions.

RESULTS

EXPRESSION OF RECOMBINANT OSTEOPONTIN FRAGMENTS IN E. COLI CELLS.

The osteopontin proteins that were used in this study include native osteopontin, recombinant human full-length osteopontin and recombinant N-and C-terminal human osteopontin fragments that would be formed following thrombin cleavage (figure 2.1). Two N-terminal fragments were used, 10N and 30N, which refer to two different splice variants of osteopontin. The 30N splice variant contains an additional 14 amino acids (NAVSSEETNDFKQE), which corresponds to exon 5.

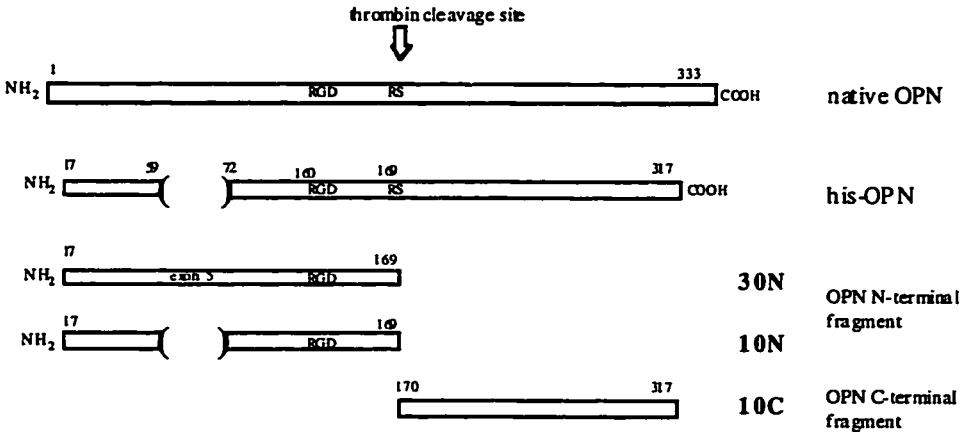

Figure 2.1. Schematic diagram of native osteopontin and human recombinant osteopontin fragments used for adhesion assays. The native, full-length osteopontin (OPN) used in these studies was purified from rat pup SMCs. The full-length human recombinant protein was prepared as a his-tagged protein (his-OPN). All other osteopontin molecules were prepared as human recombinant GST-fusion proteins. The N-and C-terminal domains are fragments that are expected to be formed following thrombin cleavage. The C-terminal osteopontin domain (10C) contains amino acids 170-317. The N-terminal osteopontin fragments (30N and 10N) includes amino acids 17-169. The two N-terminal domains are alternatively spliced. The 30N splice variant contains an additional 14 amino acids (NAVSSEETNDFKQE) which corresponds to exon 5.

Human osteopontin fragments that contain either the N-terminal domain or the C-terminal domain were amplified by PCR and cloned into the BamH1/EcoR1 sites of the expression vector pGEX-2T. The resulting plasmids contained the N-terminal domain of osteopontin including amino acids 17-169 (10N and 30N) or amino acids 170-317 (10C) fused in frame to the 3' end of the GST gene. The 30N fragment is identical to the 10N fragment except that it includes the alternate splice exon 5. Glutathione S-transferase-osteopontin fragment fusion proteins synthesized during a 2 hour induction with IPTG, were purified from bacterial lysates by affinity chromatography on glutathione-agarose beads. The pGEX-2T vector includes a thrombin cleavage site between GST and the inserted protein. Therefore, to cleave osteopontin fragments from GST, the beads were treated with biotinylated-thrombin. The biotinylated-thrombin was then separated from the osteopontin fragments by affinity chromatography on strepavidin-agarose beads. The resulting proteins were analyzed on SDS-PAGE, and are shown in figure 2.2.

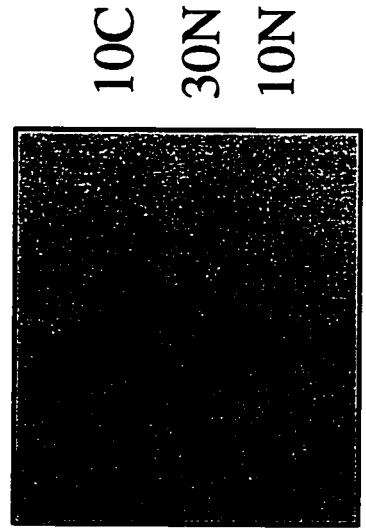

Figure 2.2. SDS-PAGE analysis of recombinant osteopontin fragments. Recombinant osteopontin fragments were expressed as GST fusion proteins in E. coli. *The GST-fusion proteins were purified with glutathione and cleaved from GST with thrombin. The resulting proteins were separated by electrophoresis on a 4-20% SDS-polyacrylamide gel and stained with coommassie blue. The C terminal osteopontin domain (10C) contains amino acids 170-317. The two alternatively spliced N-terminal fragments (30N and 10N), contain amino acids 17-169. The 30N fragment includes exon 5, while the 10N fragment lacks this exon.*

The C terminal osteopontin domain (10C) contains amino acids 170-317, and has an apparent molecular weight of 25kD. The two alternatively spliced N-terminal fragments (30N and 10N), contain amino acids 17-169. The 30N fragment includes exon 5, while the 10N fragment lacks this exon. The apparent molecular weights are 30kD and 26kD respectively. The C-terminal osteopontin domain (10C), contains a lower molecular weight protein which is most likely the result of truncated GST-10C translational products.

Cell Adhesion to Native and Recombinant Fragments of Osteopontin.

To compare the adhesive function of full length osteopontin with fragments that would be formed following thrombin cleavage at the Arg^{169}-Ser^{170} peptide bond, we performed cell attachment assays with native osteopontin, recombinant human full-length osteopontin and recombinant N-and C-terminal human osteopontin fragments. Adhesion assays were carried out with both bovine aortic endothelial cells and two different subpopulations of human melanoma cells. Bovine endothelial cells adhered to full-length native osteopontin (OPN) and both splice variants of the N-terminal osteopontin fragment (30N and 10N). There was no adhesion to the C-terminal osteopontin domain (10C) or the glutathione-S-transferase (GST) which was used as a control (figure 2.3).

We next performed adhesion assays with two subpopulations of human melanoma cell lines. The melanoma cell lines, Moα_v and Mo were derived from M21 (Chen et al., 1995). Moα_v was previously shown to express high levels of the $\alpha_v\beta_3$ integrin. Mo lacks expression of the α_v subunit, therefore these cells fail to express many of the known osteopontin receptors ($\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$). As expected, Moα_v, but not Mo cells adhered to native osteopontin (figure 2.4A). Similar results were seen with human full-length recombinant osteopontin (not shown). Surprisingly, both cell lines adhered to the 30N and 10N osteopontin fragments (figure 2.4B). There was no adhesion to the C-terminal domain or the GST-control. Because Mo cells interacted with the N-terminal domain of osteopontin and not the native protein or full-length recombinant protein, these data suggest that osteopontin adhesive interactions may be regulated by proteolytic fragmentation. Furthermore, the N-terminal region apparently contains a cryptic adhesive activity that is not exposed in the full-length molecule.

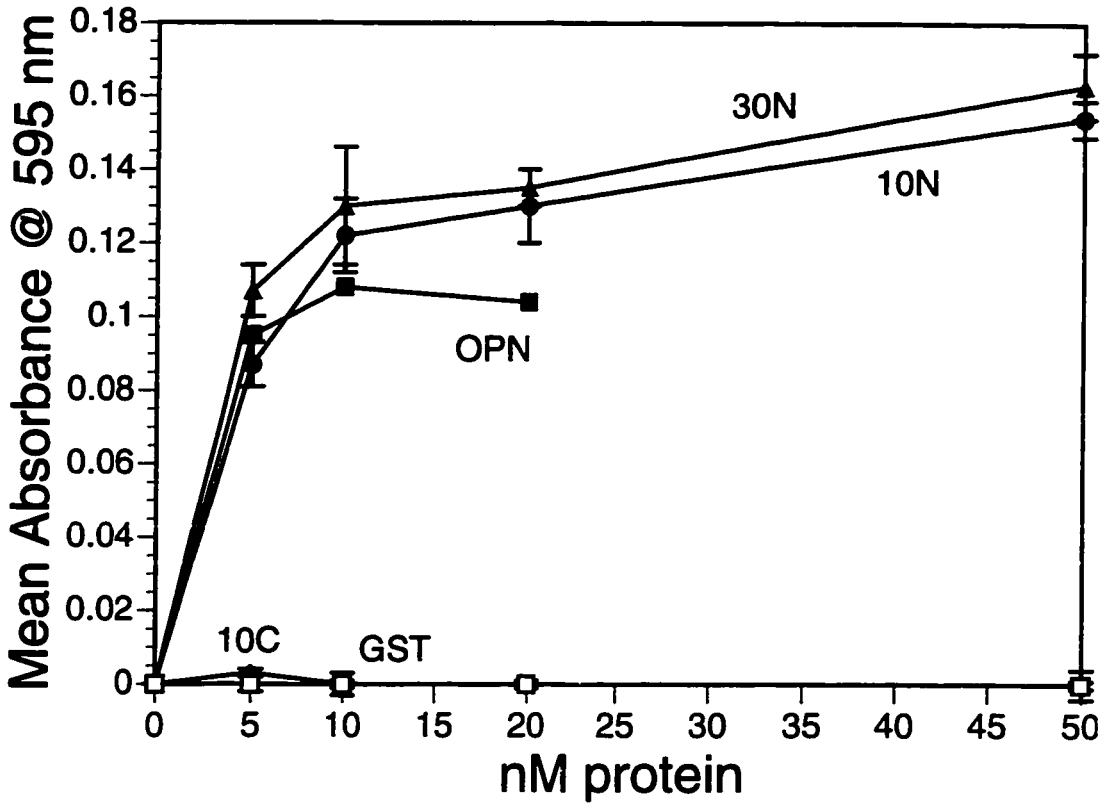

Figure 2.3. Adhesion of bovine aortic endothelial cells to native osteopontin and recombinant human osteopontin fragments. Bovine aortic endothelial cells (30,000/well) were allowed to attach for 1 hour to wells coated with the indicated concentration of native osteopontin (OPN), osteopontin recombinant fragments (30N, 10N and 10C) or glutathione-S-transferase control (GST). The attached cells were fixed, and stained with toluidine blue as described in "Materials and Methods", and the absorbance measured at 595 nm. Each data point represents the mean ± the standard deviation of triplicate samples. Non-specific cell adhesion as measured on BSA-coated wells was subtracted.

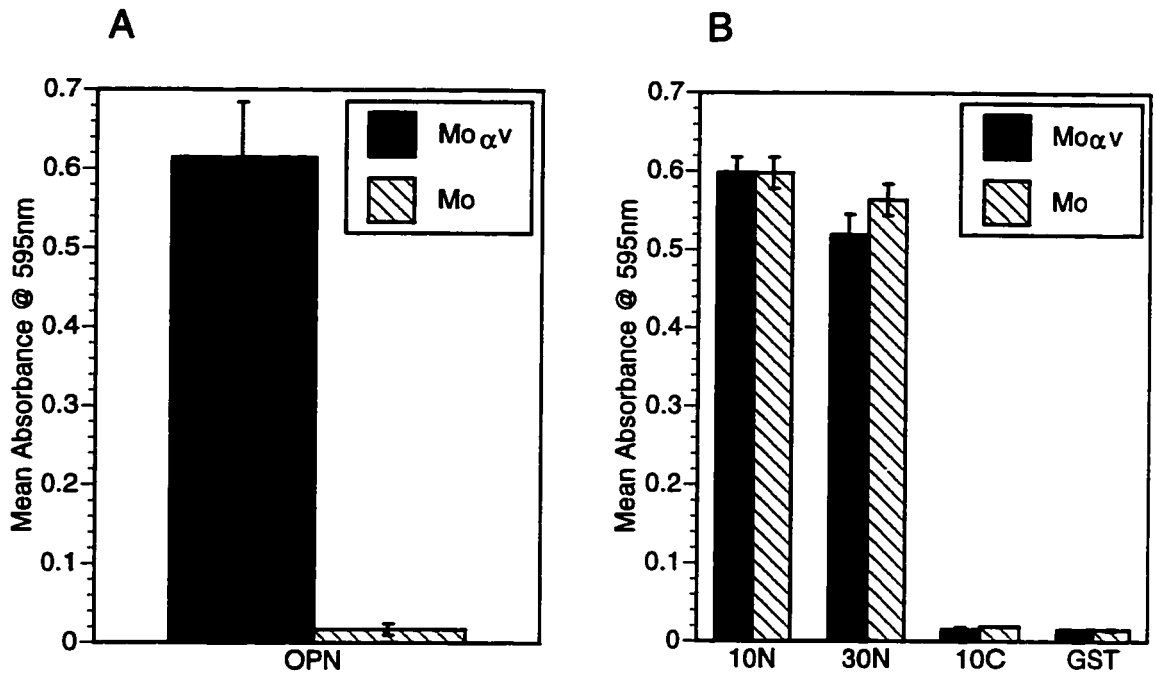

Figure 2.4. Adhesion of human melanoma cell lines, Mo and Moα_v, to native osteopontin and osteopontin recombinant fragments. Melanoma cells (100,000/well) were allowed to attach for 1 hour to wells coated with (A) 40 nM native osteopontin (OPN) or (B) 40 nM osteopontin recombinant fragments (30N, 10N and 10C). The attached cells were quantitated as described in figure 3. Each data point represents the mean ± the standard deviation of triplicate samples.

Moα_v Adhesion to Native Osteopontin and the N-terminal Osteopontin Fragment is $\alpha_v\beta_3$ and RGD dependent.

Interaction with osteopontin has been shown in many different cell types to be mediated through the $\alpha_v\beta_3$ integrin, and to be RGD dependent. Two additional osteopontin receptors, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ were first identified in human smooth muscle cells (Liaw et al., 1995) and later in human embryonic kidney cells (Hu et al., 1995). To determine the receptors mediating the interaction of Moα_v with osteopontin N-terminal domain, we performed adhesion assays in the presence of neutralizing integrin antibodies. The interaction between Moα_v and the N-terminal domain was completely blocked by both the anti-$\alpha_v\beta_3$ mAb (LM609) and an anti-α_v mAb (L230) (figure 2.5A). This indicated that the

osteopontin adhesive function was dependent on the $\alpha_v\beta_3$ integrin. Adhesion of Moα_v to native osteopontin was also $\alpha_v\beta_3$ dependent. The interaction between Moα_v and the native osteopontin was completely blocked by anti-$\alpha_v\beta_3$ mAb LM609, but not by anti-$\alpha_{IIb}\beta_3$, an irrelevant antibody control (figure 2.5B). The adherence of untreated cells and anti-$\alpha_{IIb}\beta_3$ treated cells were similar.

As expected, the interaction of Moα_v with the osteopontin N-terminal domain was mediated through the RGD sequence. GRGDSP peptide, but not GRGESP peptide inhibited Moα_v adhesion to the osteopontin N-terminal domain in a dose-dependent manner with an IC_{50} of 12 μM (figure 2.5C).

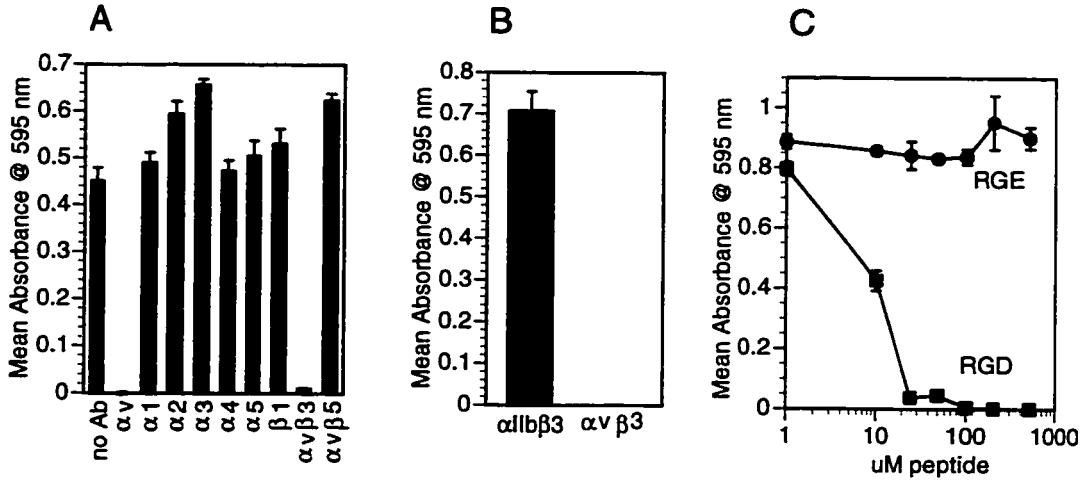

Figure 2.5. Adhesion of $\alpha_v\beta_3$ expressing melanoma cell, Moα_v, to osteopontin and the osteopontin N-terminal fragment in the presence of anti-integrin antibodies and RGD peptides. Moα_v cells were preincubated with and without neutralizing antibodies directed against the indicated integrins (A and B) or with peptides (C), for 15 minutes at 37°C before plating on wells coated with 40 nM N-terminal osteopontin fragment (10N) (A and C) or 40 nM native osteopontin (B). The monoclonal antibodies used are L230 (α_v), 5E8D9 (α_1), P1E6 (α_2), P1B5 (α_3), P4G9 (α_4), P1D6 (α_5), P4C10 (β_1), LM609 ($\alpha_v\beta_3$), P1F6 ($\alpha_v\beta_5$) and D57 ($\alpha_{IIb}\beta_3$), which was used as an irrelevant antibody control. The attached cells were quantitated as described in figure 3. Each data point represents the mean ± the standard deviation of triplicate samples

MO CELL ADHESION TO THE N-TERMINAL OSTEOPONTIN FRAGMENT IS β_1 DEPENDENT AND POORLY BLOCKED BY RGD PEPTIDES.

Mo melanoma cells lack all α_v-containing integrins (Chen et al., 1995). Since the known osteopontin receptors are $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$ or $\alpha_4\beta_1$ the interaction of Mo with the N-terminal fragment might be through $\alpha_4\beta_1$ or a novel osteopontin receptor. Binding of Mo to the N-terminal fragment was cation dependent (figure 2.6A), suggesting that this receptor was an integrin. To identify the integrin, Mo adhesion to the osteopontin N-terminal domain was carried out in the presence of integrin neutralizing antibodies. As shown in figure 2.6B, the interaction between Mo and the N-terminal osteopontin domain was entirely blocked by P4C10, a neutralizing β_1 mAb. A non-blocking β_1 antibody, LM534, failed to inhibit adhesion. The α subunit mediating this interaction was not α_1, α_2, α_3, α_4, or α_5 because blocking antibodies to these subunits had no effect on Mo adhesion (figure 2.6C).

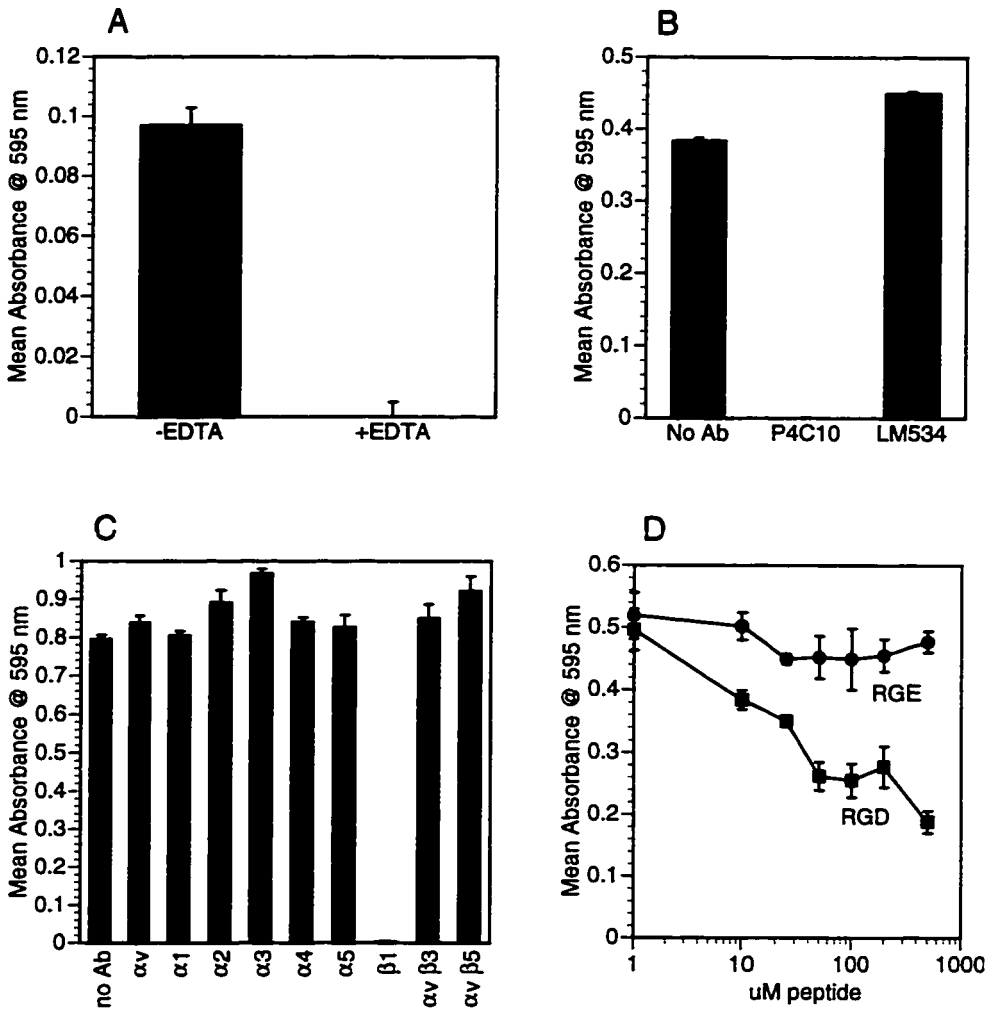

Figure 2.6. Adhesion of the α_v-null melanoma cell, Mo, to osteopontin N-terminal fragment in the presence of anti-integrin antibodies, EDTA, and RGD peptides. Mo cells were preincubated with (+EDTA) and without (-EDTA) 6.25 mM EDTA (A), the indicated neutralizing antibodies (B and C) or peptides (D), for 15 minutes at 37°C before plating on wells coated with 40 nM N-terminal osteopontin fragment (10N). The monoclonal antibodies used are L230 (α_v), 5E8D9 (α_1), P1E6 (α_2), P1B5 (α_3), P4G9 (α_4), P1D6 (α_5), P4C10 (β_1), LM609 ($\alpha_v\beta_3$) and P1F6 ($\alpha_v\beta_5$) and a non-neutralizing antibody, LM534 (β_1). The attached cells were quantitated as described in figure 3. Each data point represents the mean ± the standard deviation of triplicate samples.

The β_1 interaction with the N-terminal osteopontin fragment was only partially dependent on RGD (figure 2.6D). The GRGDSP peptide inhibited adhesion with an IC_{50} of 250 μm, which was about 20 times higher than that observed for GRGDSP inhibition of Moα_v binding to the N-terminal fragment of osteopontin. These data suggest that the N-terminal osteopontin peptide may contain an additional adhesive domain, distinct from RGD, which is recognized by the β_1 integrin on Mo cells.

IDENTIFICATION OF SURFACE EXPRESSED β_1 INTEGRINS FROM MO MELANOMA CELLS.

The antibody blocking studies suggested that the α subunit responsible for the β_1 mediated adhesion of Mo to the N-terminal fragment was not α_1, α_2, α_3, α_4, or α_5. There are four additional alpha subunits known to form heterodimers with the β_1 integrin subunits: α_6, α_7, α_8, and α_9. It is unlikely that α_6 mediates adhesion because Mo cells express extremely low levels of this integrin by FACS analysis (Table 2.1). To determine if $\alpha_8\beta_1$ was involved, we measured the adhesion of α_8 containing 293 cell transfectants to the N-terminal osteopontin fragment in the presence of neutralizing antibodies. The adhesion was completely blocked by an anti-α_v antibody suggesting that $\alpha_8\beta_1$ does not mediate adhesion by itself (not shown). To determine if the α_9 subunit was associated with surface expressed β_1 integrin, we immunoprecipitated surface biotinylated cells with P4C10, a β_1 mAb. Immunoprecipitation revealed multiple α chains of apparent molecular weight between 100 and 180Kd associated with the β_1 integrin (figure 2.7A). Two bands at molecular weight of approximately 140Kd and 115Kd were particularly abundant. These sizes correspond to the molecular weight of the α_9 and β_1 subunits, respectively. To further identify these chains, an α_9 antibody was used to immunoprecipitate surface biotinylated proteins. Figure 7B shows that the α_9 antibody immunoprecipitated abundant levels of two proteins, corresponding to α_9 and β_1 subunits, respectively (Palmer et al., 1993).

Table 2.1 Flow Cytometry Analysis of Integrin Subunit Expression on Mo and Moα_v Melanoma Cells

	Peak Fluorescence Intensity	
Antibody	**Mo**	**Moα_v**
α_1	1.90	1.65
α_2	4.14	9.12
α_3	3.10	7.74
α_4	1.62	2.64
α_5	1.26	1.30
α_6	1.83	1.72
α_9	7.02	7.24
β_1	12.14	24.27
β_6	1.02	0.98
β_3	1.21	36.49
α_v	1.17	64.60
$\alpha_v\beta_3$	1.07	26.35
$\alpha_v\beta_5$	1.07	3.88

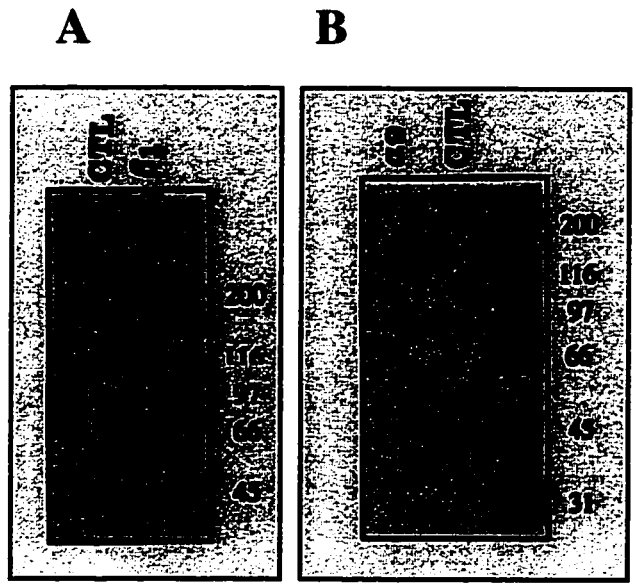

Figure 2.7. Immunoprecipitation of surface labeled proteins with β_1 and α_9 antibody. Mo cells were surface biotinylated and the cell lysate immunoprecipitated with a (A) β_1 mAb (P4C10) or (B) α_9 antibody (1057). Proteins were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was then incubated with streptavidin-biotinylated-HRP, which was visualized using chemiluminescence. The two bands at 140Kd and 115 Kd correspond to the MW of α_9 and β_1 respectively. Mouse IgG was used as a control antibody (CTL).

$\alpha_9\beta_1$ Mediates Adhesion of α_9-transfected SW480 and Mo cells to the N-terminal Domain of Osteopontin.

Because a significant level of $\alpha_9\beta_1$ was expressed on Mo cells, we next determined if $\alpha_9\beta_1$ can mediate cell adhesion to the N-terminal fragment by using a cell line that was stably transfected with α_9. SW480 cells, human colon carcinoma cells which normally do not express $\alpha_9\beta_1$ integrin, were stably transfected with a plasmid encoding for the α_9 integrin subunit, pcDNAIneoα_9. These cells have been shown to adhere to a tenascin fragment containing the third fibronectin type III repeat, while mock-transfectants do not adhere to this fragment (Yokosaki et al., 1994). The α_9-transfectants also adhered to the N-terminal osteopontin fragment (30N), and the α_9 antibody blocked this adhesion by about 60% (Figure 2.8A). This suggests that $\alpha_9\beta_1$ mediates adhesion to the N-terminal osteopontin fragment, but that other receptors are also involved.

The α_9 blocking antibody was also used in adhesion assays with Mo melanoma cells. The antibody inhibited Mo adhesion to the N-terminal osteopontin fragment by 84%. The effects of this antibody were specific because it did not interfere with non α_9-mediated adhesion to laminin (LN), collagen type I (COL I) or fibronectin (FN) (figure 2.8B). In addition, this antibody did not affect the $\alpha_v\beta_3$-mediated adhesion of Moα_v to the N-terminal osteopontin fragment (not shown).

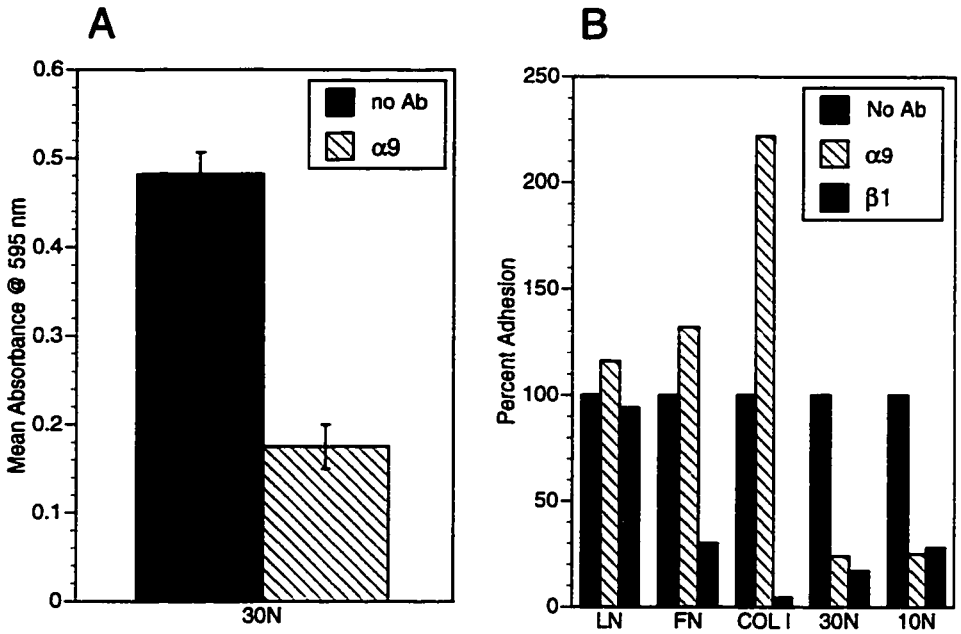

Figure 2.8. α9-mediated adhesion to N-terminal osteopontin fragment. (A) Adhesion of α_9-transfected SW480 cells to N-terminal osteopontin fragment 30N. SW480 cells, stably transfected with a plasmid encoding for α_9 integrin subunit pcDNAIneoα_9, were plated in triplicate wells (50,000 cells/well) coated with 40 nM recombinant osteopontin fragment (30N) in the absence of antibody (no Ab) or in the presence of the $\alpha_9\beta_1$ blocking antibody (Y9A2). Cells were allowed to attach for 1 hour at 37°C. Attached cells were quantified as absorbance at 595 nm of crystal violet stained wells. Each bar represents the mean (±SEM) of triplicate wells. (B) Adhesion of the α_v-null melanoma cells (Mo), to extracellular matrix molecules in the presence and absence of α_9 and β_1 neutralizing antibodies. Mo cells were preincubated with and without the neutralizing α_9 (Y9A2) and β_1 (P4C10) antibodies for 15 minutes at 37°. Cells were then plated in wells coated with laminin (LN) (10 μg/ml), fibronectin (FN) (10 μg/ml), collagen I (COL I) (10 μg/ml), 30N osteopontin fragment (40 nM) or 10N osteopontin fragment (40 nM), and allowed to attach for 1 hour. The attached cells were quantitated as described in figure 3. Each data point represents the percent adhesion compared to cells not treated with antibody.

Mo and Moα_v express similar levels of integrin receptor subunits except $\alpha_v\beta_3$

There are several possible explanations for why Mo, and not Moα_v melanoma cells interact with the N-terminal osteopontin fragment through the $\alpha_9\beta_1$ integrin. The most obvious explanation is that surface expression of $\alpha_9\beta_1$ is greater on Mo cells compared to Moα_v. FACS analysis demonstrated that this was not the case. Both melanoma cell lines contained similar levels of the α_9 integrin on their surfaces (Table 2.1). In addition, all the integrins tested had similar expression patterns in both cell lines except the α_v containing integrins. Each line expressed relatively similar levels of α_2, α_3, α_9, and β_1 integrin subunits, and low or undetectable levels of α_1, α_4, α_5, and α_6. The most significant difference between these two cells was the α_v, β_3 and the $\alpha_v\beta_3$ expression. As expected, α_v, β_3, and $\alpha_v\beta_3$ were expressed at high levels on Moα_v melanoma cells and were undetectable on Mo cells. Thus, the difference in ability of Mo and Moα_v to adhere to N-terminal osteopontin fragment is not accounted for by α_9 receptor density.

DISCUSSION

This study examined the potential role of proteolytic fragmentation on osteopontin function. We compared the N- and C-terminal GST-recombinant osteopontin fragments expected to be formed following thrombin cleavage at the Arg^{169}-Ser^{170} site, with native osteopontin in their ability to mediate adhesion of several cell lines. The results demonstrated that: 1) Osteopontin adhesive interactions may be regulated by proteolytic fragmentation such as seen with thrombin, 2) Two different splice variants of the N-terminal osteopontin fragments have identical adhesive properties, and 3) N-terminal osteopontin fragment contains two distinct integrin-binding activites. One is the RGD-dependent $\alpha_v\beta_3$-binding activity. The second is a cryptic binding activity for the $\alpha_9\beta_1$ integrin. Thus, proteolytic fragmentation may be a way of controlling or altering osteopontin's receptor specificity and thus its function.

Using N- and C-terminal recombinant fragments, we have shown that two splice variants of the N-terminal, but not the C-terminal domain can support adhesion of bovine aortic

endothelial cells and two subpopulations of human melanoma cell lines. The two subpopulations of melanoma cells differ in α_v integrin expression. Moα_v contains high levels of the $\alpha_v\beta_3$ integrin. Mo lacks expression of the α_v subunit. Because these cells lack α_v, they fail to express any of the known osteopontin receptors and do not adhere to native osteopontin. It was therefore surprising to find that the Mo cells could attach to the N-terminal osteopontin fragment. Mo cells also failed to adhere to the human recombinant full-length osteopontin indicating that the difference in adhesion of Mo cells to native osteopontin and N-terminal recombinant fragment was not simply due to glycosylation or phosphorylation. These results suggested that the adhesion of Mo cells was through a non-α_v osteopontin receptor and that a cryptic adhesive activity was exposed in the N-terminal osteopontin peptide fragment.

Further analysis demonstrated that the two subpopulations of melanoma cells adhered to the N-terminal fragment with different receptors and bound distinct adhesive domains. Moα_v cells adhered to the N-terminal fragment through the $\alpha_v\beta_3$ integrin. This interaction was RGD dependent. Mo cells, which lack α_v and fail to bind the native protein, adhered to the N-terminal fragment through the $\alpha_9\beta_1$ integrin. In addition, human urinary osteopontin that was cleaved with thrombin in situ also supported $\alpha_9\beta_1$-mediated adhesion of Mo cells (unpublished observations). The interaction of $\alpha_9\beta_1$ with the N-terminal fragment was less effectively blocked by RGD peptides suggesting there may be an additional adhesive domain distinct from RGD. A non-RGD adhesion function for osteopontin has previously been reported. A fragment from endoproteinase Arg-C digested rat osteopontin, which lacked the RGD domain, supported adhesion of human fibroblasts (van Dijk et al., 1993). However, in this study, the activity was found in the C-terminal half of the molecule, therefore this potential adhesive domain must be distinct from the $\alpha_9\beta_1$ site found in the N-terminal half of osteopontin.

The only other known ligand for $\alpha_9\beta_1$ is tenascin (TN) (Yokosaki et al., 1994). Like osteopontin, the $\alpha_9\beta_1$ binding domain in tenascin appears to be distinct from the RGD adhesion motif. (Yokosaki et al., 1994). However, Mo adhesion to the osteopontin fragment is at least partially inhibited by RGD, suggesting that either $\alpha_9\beta_1$ recognizes

osteopontin and tenascin by somewhat different mechanisms, or that other RGD-dependent receceptors are involved.

In normal adult tissue, osteopontin and α_9 are expressed on most epithelia, and could potentially colocalize (Brown et al., 1992; Palmer et al., 1993; Stepp et al., 1995; Weinacker et al., 1995). In diseased tissue, osteopontin is highly upregulated. Abundant osteopontin is found at the interface between malignant and normal tissue and at sites of inflammation and tissue remodeling (Giachelli et al., 1995; Senger et al., 1995). These are also sites where thrombin and thrombin-cleaved fragments of osteopontin are likely to be found. It would be interesting if α_9 is coordinately upregulated at these sites. If so, the ability of osteopontin to promote adhesion, migration or other cellular functions may be regulated in the presence of thrombin by exposing the cryptic domain.

Cryptic integrin-mediated binding activities have also been identified in other adhesive proteins. For example, laminin contains a cryptic peptide site that becomes functional after proteolysis and supports $\alpha_v\beta_3$-mediated adhesion of rat osteoclasts (Horton et al., 1994). Collagen also contains a cyptic site that is exposed following denaturation. Native type I collagen in its helical conformation supports $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$-meditated adhesion. These interactions are disrupted by heating or proteolysis of collagen, revealing a cryptic $\alpha_v\beta_3$ binding activity (Montgomery et al., 1994). The exposure of novel binding activities following proteolytic fragmentation is particularly relevant in remodeling tissues where proteases are active.

Both Mo and Moα_v express equal amounts of surface α_9 integrin, but only the Mo cells use this receptor for N-terminal osteopontin interactions. There are several possible explanations for this phenomenon. First, the activation state of $\alpha_9\beta_1$ on the two cell lines may differ. It is known that β1 integrins can exist in different activation states which can effect the affinity of ligand binding (Gailit and Ruoslahti, 1988; Hemler et al., 1984; Kovach et al., 1992; Masumoto and Hemler, 1993). The second possibility is that $\alpha_9\beta_1$ function could be regulated by the ligation of another integrin. For example, the interaction of $\alpha_v\beta_3$ with ligand could transmit a signal that inhibits the affinity modulation and/or function of $\alpha_9\beta_1$. This type of "cross talk" between integrins has previously been reported in several cell types (Blystone et al., 1995; Pacifici et al., 1994; Van Strijp et al., 1993).

Two previous reports have examined the functional consequences of osteopontin cleavage with thrombin. One report demonstrated that thrombin cleavage destroyed RGD-mediated adhesion (Xuan et al., 1994). In contrast, a second report showed that thrombin treatment enhanced osteopontin mediated adhesion (Senger et al., 1994). In agreement with the second report, our studies demonstrate that the N-terminal fragment that is expected to be formed following thrombin cleavage can support adhesion. It is clear from these studies as well as others, that the interaction of cells with osteopontin and osteopontin fragments is mediated through distinct receptors in different cell types. This could explain the discrepancy between different studies. Another possible explanation is that the conditions used to cleave osteopontin with thrombin may result in partial or complete proteolysis at an additional thrombin cleavage site. A second potential cleavage site is at residues Arg^{160}-Gly^{161}, which is within the RGD domain. If the RGD was destroyed by proteolytic cleavage, it is likely that $\alpha_v\beta_3$ mediated interactions would not take place.

There are several different splice variants of osteopontin. Two of the variants are due to variable usage of exon 5 which contains 14 amino acids. The functional significance of alternative splicing in this gene is not known. In this study we compared the adhesive function of recombinant osteopontin N-terminal fragments of each splice variant. Our results demonstrate that both splice variants had identical adhesive functions. Exon 5 contains the sequence NAVSSEETNDFKQE. The two serine residues are potential sites for phosphorylation or O-linked glycosylation; therefore, our data do not rule out the possibility that there is a functional difference following post-translational modification.

In conclusion, we have demonstrated that the N-terminal fragment of osteopontin contains two distinct activities. We predict from these results that osteopontin fragmentation by proteases, such as thrombin, are important in the regulation of receptor specificity and thus, the function of osteopontin.

CHAPTER 3

STRUCTURAL REQUIREMENTS FOR $\alpha_9\beta_1$-MEDIATED ADHESION AND MIGRATION TO THROMBIN-CLEAVED OSTEOPONTIN

This work in similar form was submitted for publication by Laura L. Smith and Cecilia M. Giachelli to *Experimental Cell Research*, 1998

INTRODUCTION TO CHAPTER 3

Osteopontin (OPN) is a RGD-containing phosphorylated glycoprotein thought to be important in a number of remodeling tissues including many inflammatory and fibrotic diseases. This protein is not found in the normal blood vessel wall, but is highly expressed in atherosclerotic plaques and restenotic tissue (Giachelli et al., 1993; Giachelli et al., 1995; O'Brien et al., 1994). Osteopontin is also upregulated during myocardial infarction, would repair, tumorogenesis and at sites of dystrophic calcification (Denhardt and Guo, 1993; Giachelli et al., 1995; Giachelli et al., 1997; Murry et al., 1994; O'Brien et al., 1995). *In vitro*, osteopontin has been shown to mediate adhesion and migration of a number of different cell types (D'Errico et al., 1995; Flores et al., 1996; Hu et al., 1995) including smooth muscle cells (Liaw et al., 1994; Liaw et al., 1995; Yue et al., 1994), endothelial cells (Liaw et al., 1995; Senger et al., 1996) and melanoma cells (Smith et al., 1996) through α_v-containing integrins. Osteopontin-$\alpha_v\beta_3$ interaction has also been shown to promote survival of endothelial cells (Scatena, 1997). In addition to $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_1$, the $\alpha_4\beta_1$ integrin and the CD44 receptor were also reported to interact with osteopontin (Nasu et al., 1995; Weber et al., 1996).

The RGD site in osteopontin is located in the central portion of the molecule and was shown in a number of studies to be critical for α_v-mediated interactions (Flores et al., 1996; Liaw et al., 1995; Xuan et al., 1995). In close proximity to the RGD sequence there is a thrombin cleavage site. This site is thought to be biologically important because thrombin-cleaved OPN fragments have been found *in vivo* following whole plasma blood coagulation (Senger et al., 1988; Senger et al., 1989). Since OPN and thrombin are both likely to be colocalized in remodeling tissues, it has been of great interest to understand the biological functions of OPN fragments formed following thrombin cleavage. There are several reports demonstrating that cell interaction with osteopontin can be regulated by thrombin cleavage. Senger's group showed that thrombin cleaved OPN is biologically active, and that $\alpha_v\beta_3$-mediated, RGD-dependent cell attachment and migration to the cleaved protein is substantially enhanced compared to native OPN (Senger and Perruzzi, 1996; Senger et al., 1994). In addition, we recently demonstrated that a recombinant

osteopontin fragment (30N) expected to be formed following thrombin cleavage not only biologically active, but contains cryptic activity not seen in the full length molecule (Smith et al., 1996). In that study, we found that 30N supports $\alpha_9\beta_1$-mediated adhesion of a melanoma cell line that was unable to bind native osteopontin. Although it has been shown that the RGD site is critical for the $\alpha_v\beta_3$-mediated adhesion to both the full-length OPN molecule and the recombinant N-terminal fragment, it is still not clear which adhesive domain(s) are required for $\alpha_9\beta_1$ interaction with 30N. The $\alpha_9\beta_1$-mediated adhesion could only be partially inhibited by RGD peptides suggesting that an additional site may be important (Smith et al., 1996). We hypothesized that a cryptic, non-RGD adhesive domain may exist in the full-length molecule that is exposed following thrombin cleavage, and that this domain is important for the $\alpha_9\beta_1$-mediated interaction with the N-terminal OPN fragment.

The current study was undertaken to determine which domain(s) of the N-terminal OPN are responsible for interaction with the $\alpha_9\beta_1$ integrin receptor and to determine if post-translational modifications of native OPN effect $\alpha_9\beta_1$-mediated adhesion. Additionally, we tested the effects of the N-terminal domain of OPN on the migration of $\alpha_9\beta_1$-expressing melanoma cells, since this function for $\alpha_9\beta_1$ has not yet been demonstrated. Our results indicate that $\alpha_9\beta_1$ can promote migration of melanoma cells to 30N. However, contradictory to our hypothesis, the RGD domain was required for $\alpha_9\beta_1$-mediated adhesion and migration to the N-terminal domain of osteopontin. These data suggest that the effect of thrombin cleavage is to alter the conformation of the RGD site in osteopontin such that $\alpha_9\beta_1$ is now able to recognize it.

MATERIALS AND METHODS

Cell lines - Mo and Moα_v cells were maintained in Dulbecco's Modified Eagle's Medium (Life Technologies Inc.) containing 10% fetal calf serum. Mo and Moα_v were both derived from M21 melanoma cells. One subclone, Moα_v expresses high levels of the $\alpha_v\beta_3$ integrin. Mo is a subclone expressing no detectable levels of the α_v subunit. These cell

lines were provided to us by Dr. Mark H. Ginsberg (The Scripps Research Institute) and have previously been described (Chen et al., 1995).

Antibodies - Monoclonal antibodies (mAbs) against the human $\alpha_v\beta_3$ (LM609) (Cheresh and Spiro, 1987) was purchased from Chemicon International, Inc. Monoclonal antibody against human β_1 (P4C10) (Carter et al., 1990) was purchased from Life Technologies, Inc. The neutralizing α_9 monoclonal antibody (Y9A2) (Wang et al., 1995), was provided by Dr. Dean Sheppard (Lung Biology Center, San Francisco, CA).

Adhesive Proteins - The full-length recombinant osteopontin was generated as a histidine-tagged protein as previously described (his-OPN) (Smith et al., 1996). The his-OPN construct was provided to us by Dr. Hung-Kam Cheung and Dr. Leona Ling (Biogen). Briefly, the full-length splice variant of human osteopontin (OP10) (Young et al., 1990) was cloned into pQE30 vector (Qiagen, Chatsworth, CA). *Escherichia coli* transformed with the his-OPN plasmid was grown in LB with 100 μg/ml ampicillin and induced with isopropyl-1-thio-β-D-galactopyranoside for 4 hours. The His-OPN protein was purified from the bacterial cells according to the manufacturer's instructions. (QIAexpressionist kit, Qiagen).

Recombinant osteopontin N-terminal fragment was generated by thrombin cleavage of GST-30N fusion protein as described previously (Smith et al., 1996), with several modifications. The pGEX-GST-30N fustion construct was provided to us by Dr. Hung-Kam Cheung and Dr. Leona Ling (Biogen) and the DNA sequence of verified. *Escherichia coli* JM109 cells were transformed with the plasmid and grown in LB with 200 μg/ml ampicillin. Following isopropyl-1-thio-β-D-galactopyranoside induction for 1 hour, the GST fusion proteins were purified with glutathione Sepharose beads according to the manufacturer's instructions (GST gene fusion system, Pharmacia, Piscataway, NJ). 30N OPN fragment was separated from the GST by cleaving with biotinylated-thrombin (Novagen, Madison, WI) (10 units/mg protein) at room temperature for 2 hours. Biotinylated-thrombin was then removed with UltraLink Immobilized NeutraAvidin (Pierce) and separated from the protein by centrifugation. Protein determinations were done using the Micro BCA Protein Assay Reagent Kit (Pierce).

Native osteopontin was purified from human urine. The procedure was identical to purification of osteopontin from the conditioned medium of aortic smooth muscle cell cultures as previously described (Liaw et al., 1994). Briefly, human urine was dialyzed against PBS and fractionated over a DEAE-sepharose column with a linear salt gradient (0.15M-1M NaCl). Fractions containing osteopontin were absorbed to barium citrate and eluted with 0.2 mol/L sodium citrate. The protein analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and stained with Coomassie blue. The apparent molecular weight was ~70kd in a 12.5% gel.

Tenascin fragments (TNfn3 and TNfn3-RAA) were provided to us by Dr. Kathryn L. Crossin (The Scripps Research Institute). These fragments have been previously described (Yokosaki et al., 1994).

Site-Directed Mutagenesis - The RGD sequence of GST-30N construct was mutated to RGE (30N-RGE) and RAA (30N-RAA) using the QuickChange site-directed mutagenesis kit according to the manufacturer (Stratagne Cloning Systems, La Jolla, CA). For each mutation, two oligonucleotide primers which were complimentary to the desired sequence were synthesized and HPLC purified by Gibco Life Technologies. The 5' primer 5'-CCATAAACCACACTTTCACCTCGGCC-3' and the 3' primer

5'-GGCCGAGGTGAAAGTGTGGTTTATGG-3' were used to mutate 30N to 30N-RGE. The 5' primer 5'-CCATAAACCACACTAGCAGCAGCTCGGCCATCATATG-3' and the 3' primer 5'-CATATGATGGCCGAGCTGCTAGTGTGGTTTATGG-3' were used to mutate 30N to 30N-RAA. Mutations were confirmed by complete sequencing of the full fragment for each mutant. The GST-fusion proteins containing the mutations were purified as described above for 30N.

Osteopontin expression plasmids containing a truncated C-terminal end (30N-ΔRGD) was produced by cloning polymerase chain reaction-amplified fragment into BamHI/Sma-1 sites of pGEX-2T. The primers used to amplify the fragment include the 5' primer 5'-GGCAAGCCACGTTTGGTG-3' and the 3' primer 5'-CCGGCCCGGGTCAATCATA-TGTGTCTACTGTG-3', which contains a Sma-1 site (CCCGGG) to facilitate cloning and a stop codon. The resulting plasmid has 11 amino acids truncated on the C-terminal end,

including the RGD sequence. Mutations were confirmed by complete sequencing of the full fragment and 30N-ΔRGD protein purified as described above for 30-N.

Cell Adhesion Assays - These assays were performed as described elsewhere (Liaw et al., 1994). Briefly, matrix proteins were coated onto 96-well Maxisorp microtiter plates (Nunc, Naperville, IL) overnight at 4°C and blocked one hour with PBS containing 10 mg/ml bovine serum albumin (BSA). Cells were resuspended in Dulbecco's medium containing 1 mg/ml BSA and preincubated with and without antibodies for 15 minutes at 37°C. Melanoma cells (100,000) were added to the wells and allowed to incubate for 1-2 hours at 37°C. Attached cells were stained with toluidine blue and quantitated by reading the absorbance at 595 nm. Under these conditions, absorbance was proportional to cell number (Liaw et al., 1994).

In experiments where native OPN was cleaved with thrombin *in vitro*, wells were coated with OPN overnight, followed by PBS containing 10 mg/ml BSA to block sites not occupied with osteopontin. The bound OPN was cleaved by incubating with thrombin (sigma) for 1 hour at 37°C. Prior to adding the melanoma cells, the wells were washed 2 times with PBS to remove thrombin.

Cell Migration assays - Migration assays were performed in 24-well transwell plates (Corning Costar Corporation) as previously described (Leavesley et al., 1992). Matrix proteins were coated onto the bottom of polycarbonate membranes containing 8.0 μm pores at 37°C. After 1 hour, the solution was removed and the filter air dried. The top and bottom of the filters were blocked with PBS containing 1% BSA solution for an additional hour. Cells were resuspended in Dulbecco's medium containing 1 mg/ml BSA and preincubated with antibodies for 15 minutes at 37°C. Media, with and without antibodies, was added to the lower chamber. Melanoma cells (100,000) were added to the top chamber and allowed to migrate for 16 hours at 37°C. To remove cells from the top portion of the filter, the filter was blotted gently with a cotton tipped applicator and washed with PBS several times. Cells that migrated to the bottom of the filter were fixed with methanol and stained with hematoxylin. After cutting the filter away from the tissue culture plastic, they were mounted on slides and the cells were counted in three random X400 high power fields (HPF) for each filter. Migration experiments were performed in duplicate.

RESULTS

Mo Adhesion to Thrombin-Cleaved Native Osteopontin is $\alpha_9\beta_1$-Dependent.

Previous experiments have shown that an $\alpha_9\beta_1$ expressing cell line (Mo) could adhere to a recombinant N-terminal OPN fragment (30N) through the $\alpha_9\beta_1$ integrin receptor. This cell line could not adhere to the full length osteopontin, suggesting that the $\alpha_9\beta_1$ binding site in the full length protein is cryptic. It is possible however, that the $\alpha_9\beta_1$ binding site in the recombinant N-terminal fragment is not normally accessible in the native thrombin cleaved fragments due to phosphorylation or glycosylation. To determine if native osteopontin thrombin-cleaved fragments also mediate adhesion through the $\alpha_9\beta_1$ receptor, we performed attachment assays with native human urinary osteopontin which was cleaved with thrombin. The adhesion assays were carried out with two different subpopulations of human melanoma cells, Mo and Moα_v. These cells were previously found to adhere to the recombinant osteopontin N-terminal fragment through $\alpha_9\beta_1$ and $\alpha_v\beta_3$ respectively (Smith et al., 1996). As expected, the Moα_v cells adhered to native osteopontin. In the presence of thrombin, the adhesion of Moα_v was enhanced 3-fold , which is similar to the results seen by Senger et al (Senger et al., 1994) (Figure 3.1). The Mo cells which fail to adhere to native osteopontin, attach and spread to osteopontin in the presence of increasing doses of thrombin (Figure 3.1).

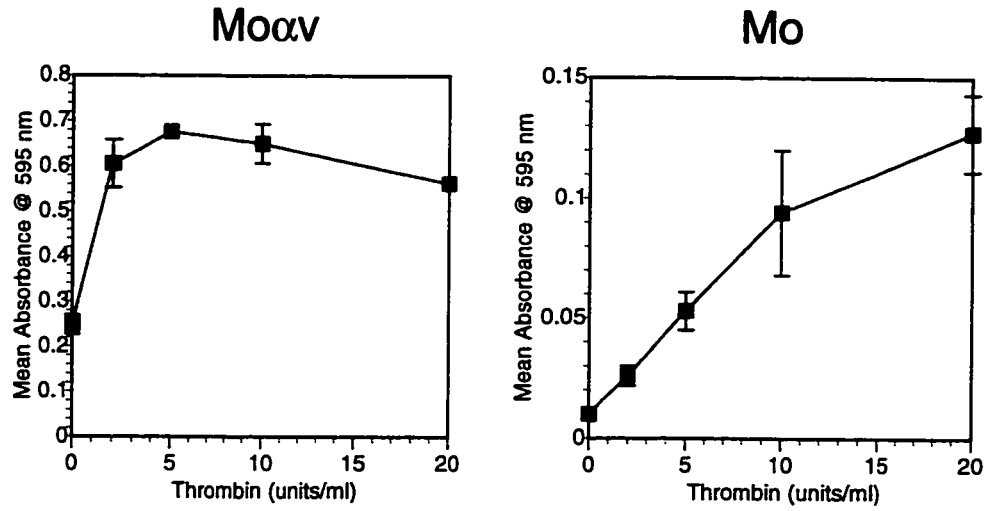

Figure 3.1. Adhesion of human melanoma cell lines, Mo and Moα_v to native human urinary osteopontin cleaved with thrombin. Wells were first coated with 0.25 μg nm native urinary OPN. The wells were then incubated with increasing doses of thrombin, or PBS as a control, for 2 hours at 37°C to cleave osteopontin. After removing thrombin by washing 2X with PBS, Mo or Moα_v melanoma cells, were allowed to attach for 2 hours. The attached cells were quantitated as described under "Experimental Procedures". Each data point represents the mean ± S.D. of triplicate samples. Nonspecific cell adhesion as measured on BSA-coated wells was subtracted.

To determine if the attachment of Mo cells to thrombin cleaved osteopontin was mediated through the $\alpha_9\beta_1$ receptor, we performed attachment assays in the presence of neutralizing α_9 and β_1 integrin blocking antibodies. The interaction between Mo cells and thrombin cleaved native osteopontin was completely blocked by both the α_9 (Y9A2) and β_1 (P4C10) mAbs, but not the $\alpha_v\beta_3$ (LM609) mAb (Figure 3.2). The α_9 and β_1 mAbs had no effect on Moα_v cell adhesion to thrombin-cleaved native OPN (not shown). In addition, the adhesion of Mo and Moα_v cells was not due to non-specific effects of thrombin, because thrombin treatment had no effect on their attachment to BSA-coated plates (not shown). These data indicate that the adhesion of Mo cells to OPN fragments formed following

thrombin cleavage is mediated through the $\alpha_9\beta_1$ integrin receptor and that differential glycosylation and phosphorylation are not important.

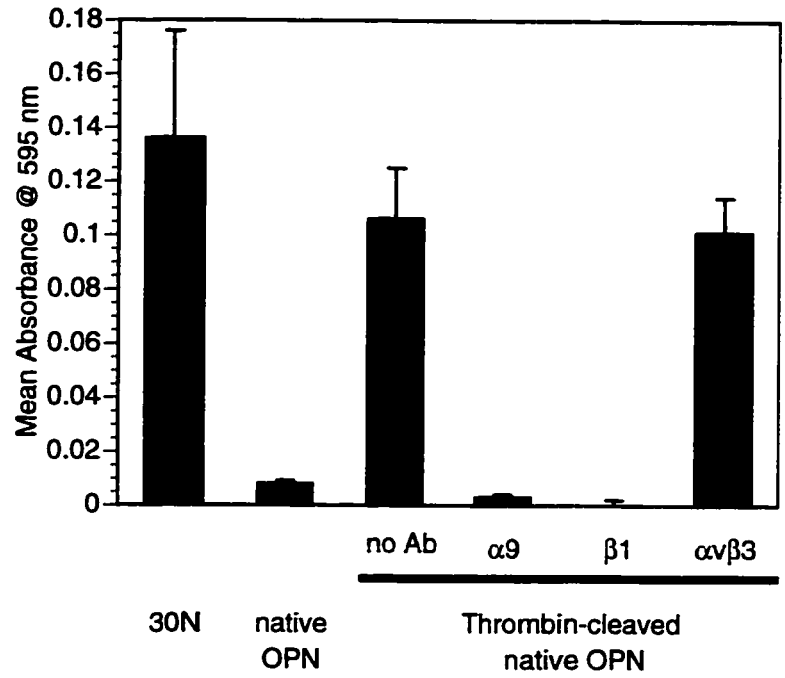

Figure 3.2. Adhesion of human melanoma cell line, Mo, to thrombin-cleaved and uncleaved native osteopontin in the presence of anti-integrin antibodies. Mo cells were preincubated with and without neutralizing antibodies directed against the indicated integrins for 15 minutes at 37°C before plating on wells coated with 150 nM N-terminal osteopontin fragment (30N), 0.25 μg native osteopontin, or native osteopontin that was cleaved with thrombin as described in the legend to figure 1. The monoclonal antibodies used are Y9A2 (α_9), P4C10 (β_1) and LM609 ($\alpha_v\beta_3$), which was used as an irrelevant antibody control. The attached cells were quantitated as described under "Experimental Procedures". Each data point represents the mean ± S.D. of triplicate samples.

Mo Cell Adhesion to the N-Terminal Osteopontin Fragment is RGD-Dependent.

Previous results showed that RGD peptides poorly blocked Mo adhesion to recombinant N-terminal OPN fragment, suggesting that the N-terminal OPN fragment may contain an additional $\alpha_9\beta_1$ adhesive domain distinct from RGD. To determine if the RGD domain is required for $\alpha_9\beta_1$-dependent 30N interaction, a mutation in the recombinant N-terminal fragment was made. Using site-directed mutagenesis, the RGD in the 30N recombinant protein was mutated to RGE (30N-RGE) (Figure 3.3 and 3.4).

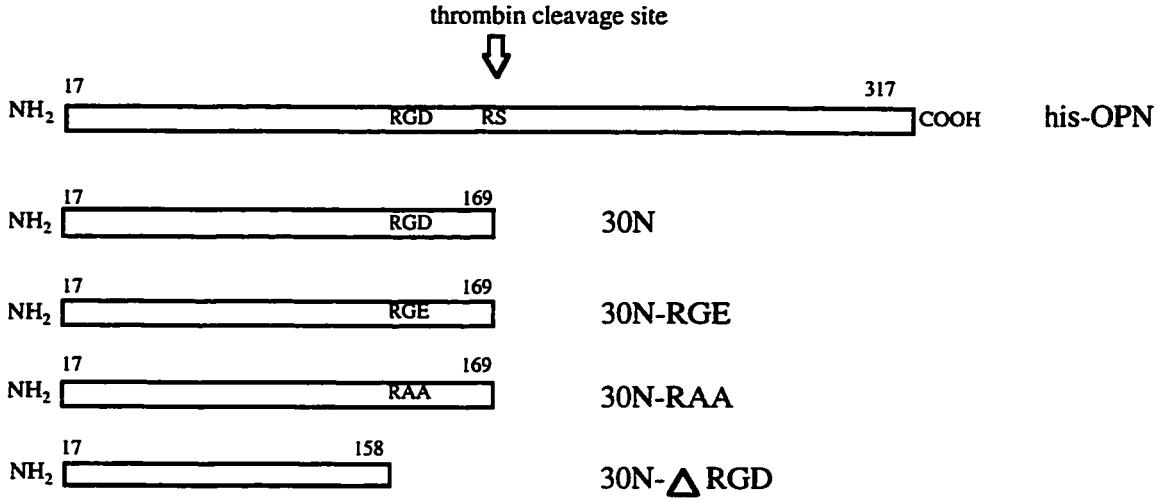

Figure 3.3. Schematic diagram of recombinant osteopontin proteins used for adhesion and migration assays. The full-length recombinant protein was prepared as a His-tagged protein (His-OPN). All other osteopontin molecules were prepared as human recombinant GST-fusion proteins and the GST subsequently cleaved off. The N-terminal fragments: 30N, 30N-RGD, and 30N-RAA, includes amino acids 17-169. The 30N-Δ RGD is a truncated version of the 30N that eliminates the last 11 amino acids including the RGD site.

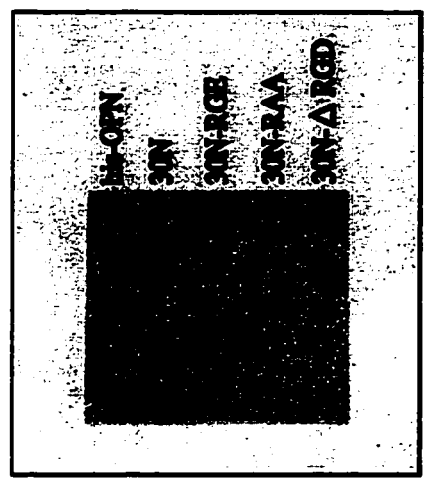

Figure 3.4. SDS-PAGE analysis of recombinant osteopontin fragments. The wild type and mutant recombinant osteopontin fragments were expressed as GST-fusion proteins in E. coli. *GST fusion proteins were purified on glutathine and cleaved from GST with biotinylated thrombin. The proteins were separated by electrophoresis on a 15% SDS-polyacrylamide gel and stained with colloidal blue stain. The wild type 30N fragment and the 30N point mutants contain amino acids 17-169. The 30N-Δ RGD, is a truncated version of 30N that lacks the last 11 amino acids.*

The 30N-RGE mutant failed to support the adhesion of Moα_v cells (Figure 3.5). This result was expected since RGD peptides completely block the adhesion of these cells to the 30N fragment (Smith et al., 1996) and the adhesion of HFL-1 cells to thrombin cleaved OPN (Senger et al., 1994). The adhesion of Mo cells to 30N-RGE was reduced by 50%, suggesting that this domain was also important for $\alpha_9\beta_1$-mediated interactions with the 30N-OPN fragment (Figure 3.5). The residual adhesion to 30N-RGE could be blocked with neutralizing α_9 and β_1 antibodies (Figure 3.6).

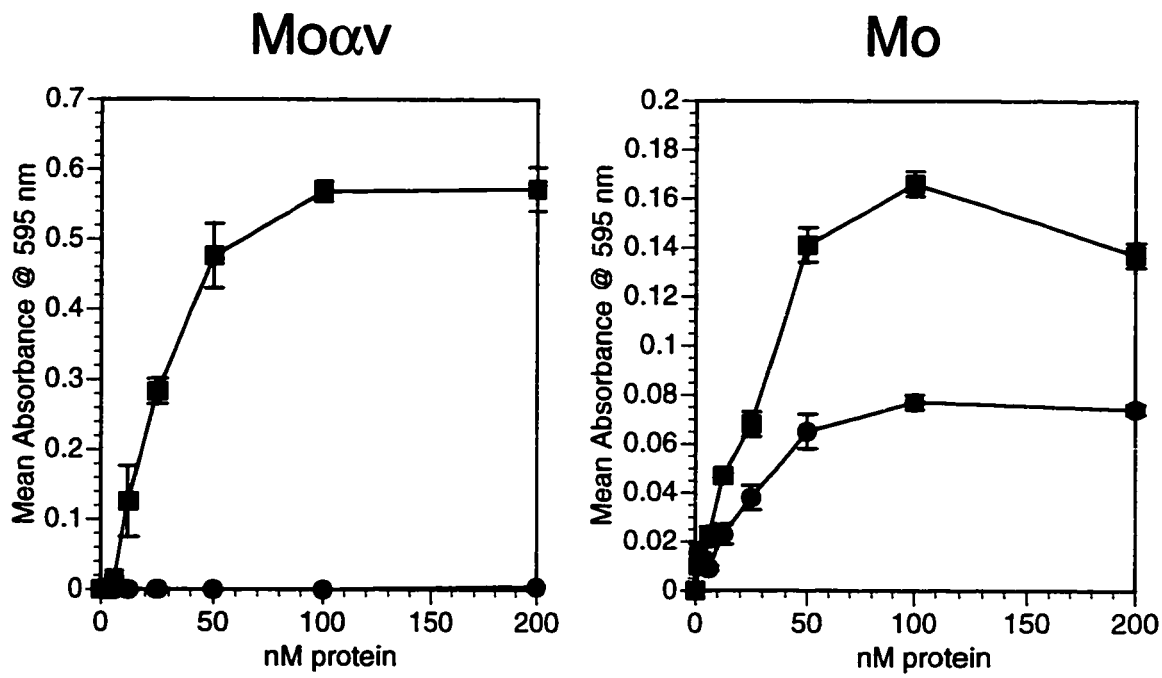

Figure 3.5. Adhesion of human melanoma cell lines, Mo and Moα_v to N-terminal osteopontin fragments with or with an RGE mutation. Melanoma cells, Moα_v and Mo, were allowed to attach for 2 hours to wells coated with 200 nM of the wild-type N-terminal OPN fragment (30N) (■) or 200 nM of the mutant 30N fragment in which the RGD site has been mutated to RGE (30N-RGE) (●). The attached cells were quantitated as described under "Experimental Procedures". Each data point represents the mean ± S.D. of triplicate samples.

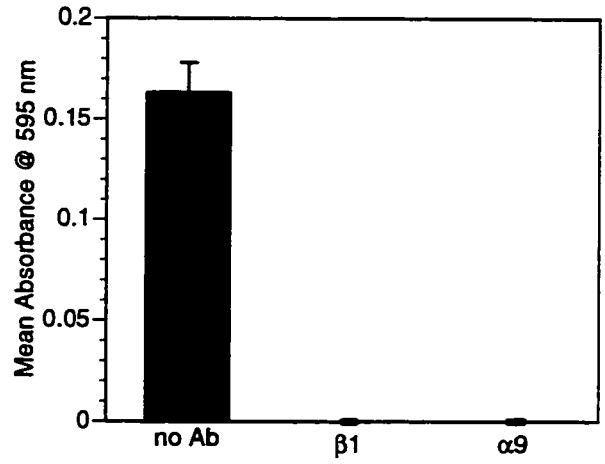

Figure 3.6. Adhesion of melanoma cell line, Mo, to 30N-RGE osteopontin fragment in the presence of anti-integrin antibodies. Mo cells were preincubated with and without neutralizing antibodies directed against the indicated integrins for 15 minutes at 37°C. The cells were then plating on wells coated with 100 nM N-terminal OPN fragment containing a RGE mutation (30N-RGE). The monoclonal antibodies used are P4C10 (β_1) and Y9A2 (α_9). Attached cells were quantitated as described under "Experimental Procedures". Each data point represents the mean ± S.D. of triplicate samples.

There are several explanations for the residual adhesion of Mo cells to 30N-RGE. One possible explanation is that the $\alpha_9\beta_1$ has enough affinity for the RGE sequence to support an interaction. If this were the case, mutation from RGD to RAA or a RGD deletion should eliminate all adhesion. Alternatively, the $\alpha_9\beta_1$ receptor may recognize two distinct sites on 30N: the RGD site and an unknown site. If so, mutation of RGD to RAA should still result in 50% attachment. To differentiate between these possibilities, several mutations were made. One mutation in the 30N fragment changes the RGD to RAA (30N-RAA). An additional mutant was made which lacks the last 11 amino acids, including the RGD (30N-ΔRGD). A schematic diagram and SDS gel analysis of these proteins are shown in figures 3.3 and 3.4 respectively. The 30N-RAA protein has an apparent molecular weight which is slightly higher then the wild type protein. The reason for the difference is not known. By sequence analysis, 30N-RAA was identical to 30N except for the appropriate mutation. A second protein preparation was prepared from a plasmid containing an independently derived 30N-RAA insert. This plasmid also contained the appropriate sequence and had an aberrant molecular weight on an SDS-gel identical to the first preparation. Unexpectedly,

both mutations eliminated Mo attachment suggesting that the RGD site is required for $\alpha_9\beta_1$ interactions and that it is unlikely that there are two different $\alpha_9\beta_1$ binding sites (figure 3.7).

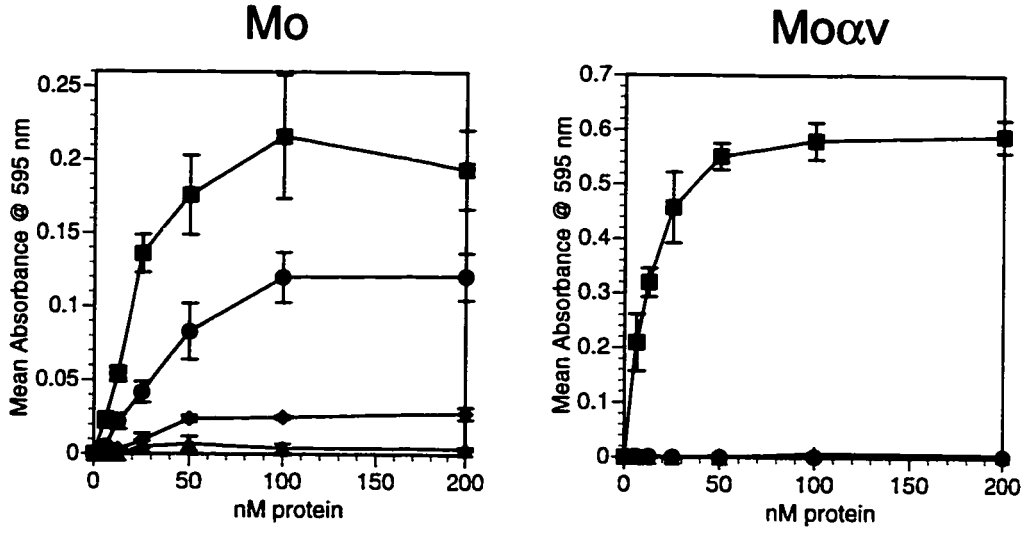

Figure 3.7. Adhesion of human melanoma cell lines, Mo and Moα$_v$, to N-terminal osteopontin fragments with and without mutations at the RGD site. Mo and Moα$_v$ melanoma cells were allowed to attach for 2 hours to wells coated with 200 nM of the following ligands: wild-type N-terminal OPN fragment (30N) (■), mutant 30N fragment in which the RGD site has been mutated to either RGE (30N-RGE) (●), or RAA (30N-RAA) (◆), or a truncated fragment in which the last 11 amino acids, including the RGD site have been eliminated (30N-Δ RGD) (▲). The attached cells were quantitated as described under "Experimental Procedures". Each data point represents the mean ± S.D. of triplicate samples.

Mo Cell Migration to N-Terminal Osteopontin Fragment is $\alpha_9\beta_1$- and RGD-Dependent.

The $\alpha_9\beta_1$ integrin has been reported to mediate cell spreading, attachment, and proliferation (Yokosaki et al., 1996). It has not, however, been shown to stimulate cell movement. Because the N-terminal OPN fragment is capable of promoting $\alpha_v\beta_3$-mediated migration (Senger and Perruzzi, 1996), we were interested in determining if $\alpha_9\beta_1$ could also mediate migration. In a transwell migration assay, Mo cells migrated across the filter to the top of the other side which had been coated with 30N (figure 3.8). Both α_9 and β_1 neutralizing antibodies blocked the migration completely indicating that $\alpha_9\beta_1$ is necessary for cell

motility. The cells did not however, migrate to full-length his-OPN. The cells also failed to migrate to the 30N-RAA mutant OPN indicating that the RGD site is necessary for cell motility.

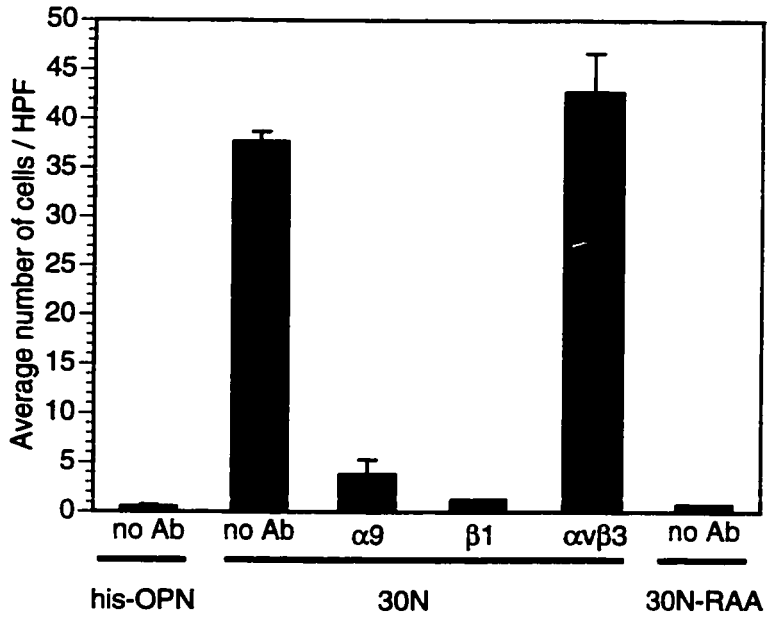

Figure 3.8. Migration of Mo cells to the full-length recombinant osteopontin and N-terminal osteopontin fragments with or without the presence of anti-integrin antibodies. Mo melanoma cells were preincubated with and without neutralizing antibodies directed against the indicated integrins for 15 minutes at 37°C. The cells were then added to the top of transwells and allowed to migrate toward the lower portion of the filter which was coated with ligands. The lower portion of the filters were coated with either 100 nM recombinant full-length osteopontin protein (his-OPN), 100 nM N-terminal OPN fragment (30N) or 100 nM N-terminal OPN fragment containing a RAA mutation (30N-RAA). The monoclonal antibodies used are P4C10 (β_1), Y9A2 (α_9) and 27.1 ($\alpha_v\beta_3$), which was used as an irrelevant antibody control. After 16 hours, the cells that migrated to the lower side through the porous filter were quantitated by counting the cells in high power fields (HPF X400) as described under "Experimental Procedures". Each data point represents the mean ± S.D. of duplicate samples.

N-Terminal Osteopontin Fragment and Tenascin Support Adhesion and Migration of Mo Cells Through Different Adhesive Domains.

The only other known ligand for $\alpha_9\beta_1$ is tenascin. Tenascin was previously shown to support $\alpha_9\beta_1$-mediated adhesion of RD, Tera 2 cells and α_9-transfected SW480 cells through the FN type III repeat. This interaction was found to be independent of the RGD

domain (Yokosaki et al., 1994). In contrast, our results show that the RGD domain is necessary for the $\alpha_9\beta_1$-mediated adhesion and migration of Mo cells to 30N. To confirm the non-RGD mediated interaction of $\alpha_9\beta_1$ with TN using Mo cells, attachment assays were performed with the tenascin FN type III fragment (TNfn3) and the tenascin FN type III-RAA mutant (TNfn3-RAA). The Mo cells could adhere to the wild type TNfn3 as well as the TNfn3-RAA mutant and the adhesion could be blocked with α_9 and β_1 neutralizing antibodies (figure 3.9A). In addition to adhesion, we also found that Mo cells migrated to TNfn3-RAA mutant (figure 3.9B). As expected, the Moα_v cells which attach to TNfn3

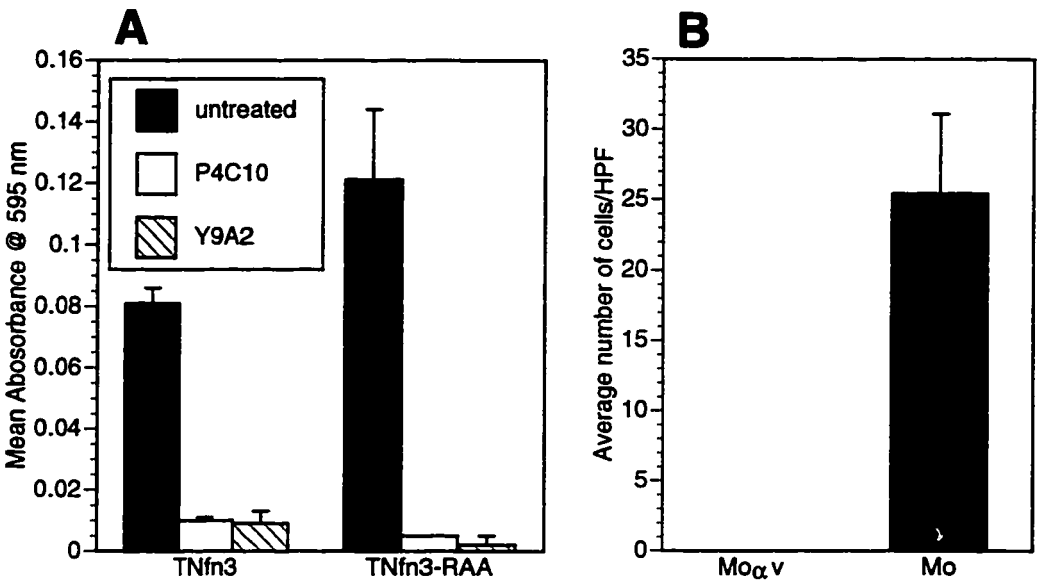

Figure 3.9. Adhesion and migration of Mo cells to recombinant TNfn3 with or without mutations at the RGD site. Panel A *shows the result of a cell adhesion assay to recombinant tenascin fragments. Mo melanoma cells were preincubated with and without neutralizing antibodies directed against the indicated antibodies for 15 minutes at 37°C. The cells were then allowed to attach for 2 hours to wells coated with 10 µg/ml of the recombinant tenascin fragment containing the third fibronectin type III repeat (TNfn3) or the same fragment in which the RGD site has been mutated to RAA (TNfn3-RAA). The monoclonal antibodies used are P4C10 (β_1) and Y9A2 (α_9). The attached cells were quantitated as described under "Experimental Procedures". Each data point represents the mean ± S.D. of triplicate samples.* Panel B *shows the result of a cell migration assay to recombinant tenascin fragment containing an RAA mutation. Mo and Moα_v melanoma cells were added to the top of transwells and allowed to migrate toward the lower portion of the filter which was coated with 10 µg/ml TNfn3-RAA. After 16 hours, the cells that had migrated to the lower side through the porous filter were quantitated by counting the cells in high power fields (HPF X400) as described under "Experimental Procedures". Each data point represents the mean ± S.D. of duplicate samples.*

through the RGD domain via the $\alpha_v\beta_3$ receptor were unable to migrate to TNfn3-RAA mutant. These results confirm previous studies showing that $\alpha_9\beta_1$-mediated interaction with TN is non-RGD dependent (Yokosaki et al., 1994). They also demonstrate that the $\alpha_9\beta_1$ interactions with the N-terminal fragment of OPN and TN are through different adhesive domains.

DISCUSSION

In this report, we identified the sequences in the N-terminal domain of osteopontin important for $\alpha_9\beta_1$ interactions and examined the effect of post-translational modification of native OPN on $\alpha_9\beta_1$-mediated adhesion. The results demonstrate that: 1) like recombinant 30N, native OPN cleaved with thrombin *in vitro* supports $\alpha_9\beta_1$-mediated adhesion; 2) $\alpha_9\beta_1$ mediates the migration of Mo cells to the N-terminal OPN fragment, a function not yet described for the $\alpha_9\beta_1$ integrin; and 3) in striking contrast to interactions with tenascin, the RGD sequence is required for $\alpha_9\beta_1$ interaction with 30N osteopontin.

Using site directed mutagenesis, we made mutations in the RGD site of 30N to RGE and RAA. We also made a mutant protein truncated at the C-terminal end such that the last 11 amino acids, including the RGD site, were eliminated. We compared these recombinant mutant proteins with wild type 30N in their ability to mediate adhesion of an $\alpha_9\beta_1$-expressing cell line. The results demonstrate that the RGD site is required for $\alpha_9\beta_1$-mediated interactions. In addition, we found that $\alpha_9\beta_1$-expressing melanoma cell line, Mo, could migrate to 30N. The migration was both $\alpha_9\beta_1$- and RGD-dependent. Using a 30N-RGE recombinant protein, we demonstrated that $\alpha_9\beta_1$-mediated adhesion was 50% of that seen using the wild type 30N protein. This finding is in agreement with our previous studies that showed that RGD peptides were capable of only partially inhibiting the adhesion of Mo cells to the N-terminal fragment (Smith et al., 1996).

Based on these data, we hypothesized that the N-terminal domain may contain an additional adhesive site, distinct from the RGD that is important for $\alpha_9\beta_1$-mediated interactions. This hypothesis was appealing because $\alpha_9\beta_1$ can not recognize the full-length OPN molecule

indicating that the $\alpha_9\beta_1$ binding site was cryptic in the native protein, and would account for the gain of activity seen following thrombin cleavage. An alternative explanation of the results is that the RGD sequence was the only $\alpha_9\beta_1$ binding site on 30N and that attachment to the 30N-RGE mutant was due to residual recognition of this site by $\alpha_9\beta_1$. To differentiate between these two possibilities, we made more drastic mutations in the RGD site by mutating RGD to RAA or by eliminating the RGD completely. We reasoned that if an adhesive domain distinct from the RGD was important for $\alpha_9\beta_1$-mediated interactions, then these mutants would still support adhesion of Mo cells. Unexpectedly, we found that the Mo cells failed to adhere to 30N-RAA and 30N-ΔRGD, indicating that contradictory to our first hypothesis, the RGD domain is required for all the $\alpha_9\beta_1$-mediated adhesion to N-terminal osteopontin fragment.

Our studies also suggest that the interaction between $\alpha_9\beta_1$ and the RGD site on 30N is distinct from the interaction between $\alpha_v\beta_3$ and the RGD site. For example, changing the aspartic acid to a different negatively charged amino acid (RGD to RGE), has dramatic consequences on $\alpha_v\beta_3$ interactions and eliminated all adhesive activity. However the $\alpha_9\beta_1$-mediated interaction was only reduced by 50%, indicating that the amino acid requirements for integrin engagement are much less stringent.

The results of these experiments do not explain why only the proteolytic fragments of OPN and not the full-length molecule can support $\alpha_9\beta_1$-mediated activities. It is possible that sequences exposed following thrombin cleavage are necessary for the $\alpha_9\beta_1$-RGD interaction. An alternative explanation is that the RGD site may take on a distinct conformation in the thrombin cleaved fragment compared to the full-length molecule, and that the $\alpha_9\beta_1$ integrin may be particularly sensitive to these changes. This is a possibility since the RGD domain is just 6 amino acids away from the thrombin cleavage site. The RGD site in the native protein is predicted to be found in a loop between two beta-sheets (Craig et al., 1989; Prince, 1989), as is the RGD site in many other proteins (Ruoslahti, 1996). This restrained conformation may not be conducive for $\alpha_9\beta_1$ interactions. Following thrombin cleavage, the restrained nature of this domain may be lost and the binding to the $\alpha_9\beta_1$ integrin promoted. The $\alpha_v\beta_3$ binding may also be effected by the conformation change since $\alpha_v\beta_3$-mediated attachment is enhanced following thrombin

cleavage. It is interesting that the only other ligand for $\alpha_9\beta_1$, tenascin, contains an RGD site in the loop regions of the fn type III repeat, yet the $\alpha_9\beta_1$ interaction with tenascin is though a non-RGD mechanism (Yokosaki et al., 1994).

In addition to its adhesive properties, osteopontin also promotes migration of a variety of cell types through the $\alpha_v\beta_3$ integrin (Liaw et al., 1994; Liaw et al., 1995; Liaw et al., 1995; Yue et al., 1994). $\alpha_v\beta_3$ is also important for cell migration to thrombin cleaved OPN fragments (Senger et al., 1996; Senger and Perruzzi, 1996). Although $\alpha_9\beta_1$ integrin has not been reported as a migratory receptor, the expression of $\alpha_9\beta_1$ near migrating cells in the cornea would suggest that migration could potentially be a $\alpha_9\beta_1$-mediated function (Stepp and Zhu, 1997). To determine the migratory function of the N-terminal fragment of OPN and $\alpha_9\beta_1$, we tested the ability of 30N to promote the migration of Mo cells in a transwell migration assay. We show that recombinant 30N protein could stimulate migration of Mo cell in a $\alpha_9\beta_1$- and RGD-dependent manner. Consistent with our previous findings for $\alpha_9\beta_1$-mediated adhesion, the full-length protein failed to promote $\alpha_9\beta_1$-mediated migration, suggesting that this activity is only exposed following thrombin cleavage.

The only other known ligand for $\alpha_9\beta_1$ is tenascin. The $\alpha_9\beta_1$ binding site in tenascin appears to be distinct from the RGD site since mutations in this domain of the TN-fn type III fragment did not effect $\alpha_9\beta_1$-mediated cell adhesion (Yokosaki et al., 1994). To confirm these results in our system and to compare the mechanisms of Mo attachment to the two $\alpha_9\beta_1$ ligands, we performed adhesion assays in the presence of recombinant TN-fn3 fragments that contain either RGD or fragments in which the RGD site has been changed to RAA. As expected from previous reports, we found that Mo cell could adhere to TNfn3 protein and the TNfn3-RAA mutant through the $\alpha_9\beta_1$ integrin. In addition, the TNfn3-RAA mutant could support migration of Mo cells where the similar mutation in 30N could not. These studies strongly suggest that $\alpha_9\beta_1$ recognizes OPN and TN through different mechanisms, potentially involving unique ligand binding sites on $\alpha_9\beta_1$..

Although the ability of one integrin receptor to interact with several distinct binding sites is not commonly observed, at least two other integrins have been reported to recognize

distinct sequences. The $\alpha_{IIb}\beta_3$ integrin, which is found on platelets, can attach to many different matrices through the RGD sequence, but can also attach to fibrinogen via the dodecapeptide (Farrell and Thiagarajan, 1994; Haas and Plow, 1994). The $\alpha_4\beta_1$ integrin recognizes the CS-1 sequence in fibronectin. However, under conditions of integrin activation, the $\alpha_4\beta_1$ can also recognize the RGD domain (S'Anchez Aparicio et al., 1994). We were able to rule out the possibility that the differential mechanism of $\alpha_9\beta_1$-mediated attachment to OPN and TN is due to a difference in $\alpha_9\beta_1$ activation state since the same cells were used for both ligands.

The adhesive and migratory properties of the proteolytic fragments of OPN suggest a potential role in remodeling tissues, inflammation and tumorogenesis, where thrombin is likely to be co-localized with osteopontin. In addition to osteopontin, $\alpha_9\beta_1$ has also been shown to localize to some remodeling tissues. For example, $\alpha_9\beta_1$ is upregulated during epithelial regeneration following debridement in the cornea (Stepp and Zhu, 1997) and is upregulated in patients with light-chain deposition disease and amyloid light-chain amyloidosis (Turbat Herrera et al., 1997). In addition, both $\alpha_9\beta_1$ and osteopontin are localized to the ductus arteriosus during the remodeling process required for ductus closure (Clyman et al., 1996; Thayer et al., 1995).

In conclusion, we have demonstrated that the N-terminal fragment of osteopontin supports $\alpha_9\beta_1$-mediated adhesion and migration through the RGD sequence. This mechanism of interaction is distinct from that of tenascin, which interacts with $\alpha_9\beta_1$ through a non-RGD domain.

CHAPTER 4

OSTEOPONTIN DOES NOT INTERACT WITH SOLUBLE CD44-IG PROTEIN AND SEVERAL CD44 SPLICE VARIANTS

This work in similar form is being prepared for publication by Laura L. Smith, Brad W. Greenfield, Alejandro Aruffo, and Cecilia M. Giachelli.

INTRODUCTION TO CHAPTER 4

Osteopontin (OPN) is a multifunctional secreted glycoprotein implicated in a number of diseases associated with remodeling (Denhardt and Guo, 1993; Giachelli et al., 1993; Giachelli et al., 1995; Giachelli et al., 1994; Giachelli et al., 1997; Giachelli et al., 1995; Murry et al., 1994; O'Brien et al., 1995). In vitro, osteopontin has been shown to be an adhesive protein and a migratory stimulus for a variety of different cell types (D'Errico et al., 1995; Flores et al., 1996; Hu et al., 1995; Smith et al., 1996) including smooth muscle cells (SMC) and endothelial cells (Liaw et al., 1994; Liaw et al., 1995; Yue et al., 1994). Liaw et al has shown that the interaction of these cell types with OPN is mediated through the $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ integrin receptors in an RGD-dependent manner (Liaw et al., 1995). In addition to α_v-containing integrins, $\alpha_4\beta_1$ was shown to mediate adhesion of a macrophage cell line (Nasu et al., 1995), and the $\alpha_9\beta_1$ integrin was shown to interact with the N-terminal fragment of osteopontin following thrombin cleavage (Smith et al., 1996).

More recently, Weber et al, identified osteopontin as a ligand for CD44, a non-integrin, cell surface glycoprotein (Weber et al., 1996). CD44 is expressed on a broad range of normal and malignant tissues (Hofmann et al., 1991; Matsumura and Tarin, 1992). It plays a role in cell adhesion, cell migration, lymphocyte activation, lymphocyte homing and cancer metastasis (Aruffo et al., 1990; Gunthert et al., 1991; Jalkanen et al., 1986; Lesley et al., 1993; Taher et al., 1996). The primary receptor for CD44 is hyaluronic acid (HA) (Aruffo et al., 1990; Culty et al., 1990; Miyake et al., 1990), however it has also been shown to interact with fibronectin, collagen, and heparin-binding growth factors (Bennett et al., 1995; Jalkanen and Jalkanen, 1992; Tyrrell et al., 1993). The multifunctional property of CD44 is probably due to the many different variant isoforms that exist. Human CD44 contains 20 exons and at least 12 exons can be alternatively spliced (Screaton et al., 1992). The most abundant isoform is the hematopoietic variant (CD44H or CD44s) which is widely distributed (Stamenkovic et al., 1989). This variant, which is 85-95 kD, lacks all the variable exons. Larger variant forms are generated by alternative splicing (CD44v), and some of these variants seem to correlate with invasive and metastatic capacity of tumor cells in vivo (Gunthert, 1996; Gunthert et al., 1991; Koopman et al., 1993; Matsumura and

Tarin, 1992; Tanabe et al., 1993; Terpe et al., 1994). In addition to alternative splicing, differential glycosylation can also lead to variants containing chondroitin sulphate and heparin sulfate (Cooper and Dougherty, 1995; Freed et al., 1989; Jackson et al., 1995; Labarriere et al., 1994).

CD44-osteopontin interactions could potentially be very important during inflammation, tumorogenesis, and tissue remodeling. We are particularly interested in the potential role of OPN interactions with CD44 in vascular disease because both molecules are expressed in remodeling vascular tissue (Giachelli et al., 1993; Giachelli et al., 1995; Giachelli et al., 1995; Jain et al., 1996) and in angiogenic vessels (Giachelli et al., 1993; Griffioen et al., 1997). In addition, both molecules have been implicated in adhesive and migratory functions; two processes important for vascular remodeling.

Although the potential interaction of vascular smooth muscle cell (SMC) CD44 with OPN has not been investigated, our own studies have demonstrated that SMCs, which express high levels of CD44 (Jain et al., 1996), do not interact with osteopontin through the CD44 receptor. Attachment and migration of SMCs to OPN can be blocked with $\alpha_v\beta_3$-integrin neutralizing antibodies and RGD peptides suggesting that this interaction is mediated through integrins (Liaw et al., 1994; Liaw et al., 1995). Moreover, adhesion of SMC to OPN does not take place in the absence of cations (Liaw et al., 1995); a requirement for integrin interactions, but not CD44 interactions. The lack of CD44-mediated attachment of SMC to OPN may not be all that surprising since there are multiple CD44 isoforms that are differentially expressed on various cell types. It is possible that only particular splice variant(s) of CD44, not expressed on vascular SMCs can interact with OPN.

This study was undertaken to determine if osteopontin is a ligand for the standard CD44 receptor and some of the other CD44 variants thought to be upregulated during disease. We used the standard form of CD44-immunoglobulin (CD44-hIg) fusion proteins and several CD44 splice variants in enzyme-linked immunosorbant assays (ELISA) to examine the CD44-osteopontin interactions. We found that although the CD44-hIg proteins could interact with HA as expected, there was no interaction between CD44H, CD44E, CD44v3, v8-v10 or CD44v3 with osteopontin.

MATERIALS AND METHODS

Cell Lines- COS cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Gaithersburg, MD) with 7% FBS, penicillin (100 u/ml), streptomycin (100 μg/ml) and 2mM L-glutamine. WEHI-3B cells were provided to us by Dr. Alan Sartorelli (New Haven, CT) and were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal calf serum.

Construction of CD44-Ig Expression Vectors- CD44-hIg constructs hCD44H-hIg and hCD44H-R41A-hIg mutant were previously described (Peach et al., 1993). CD44-hIg constructs hCD44E-hIg and hCD44 V3,V8-V10-hIg were also previously described (Bennett et al., 1995). CD44 exon V3 construct was generated by PCR using CD44 V3-FP-SpeI: ACTAGTACGTCTTCAAATACCATCTCAG and CD44 V3-RP-BamHI: GGGATCCAGGGTGCTGGAGATAAAATCTTC. PCR reaction conditions were as follows: 94°C for 5 minutes, with 35 cycles of 94°C for 30 seconds, 57°C for 1 minute, and 72°C for 1 minute and 45 seconds. PCR products were purified with Qiaquick spin PCR purification kit (Qiagen Corp., Santa Clarita, CA). Purified products were digested with restriction enzymes SpeI and BamHI (Boehringer Mannheim Corp., Indianapolis, IN), gel purified and ligated into SpeI/BamHI cut vector CDM7B- with CD5 signal sequence 5' and human Ig 3' of CD44 insert as described (Bennett et al., 1995). All DNA constructs were sequenced for correct inserts.

CD44-hIg Fusion Protein Expression- Transient expression of fusion proteins in COS cells is previously described (Aruffo et al., 1990). Fusion protein supernatents were purified over protein-A sepharose column, eluted in 4M imidizole with 1mM each $MgCl_2$ and $CaCl_2$. Eluted protein was dialyzed extensively in 1x PBS. Protein concentrations were determined using Pierce BCA Assay as described by manufacturer (Pierce, Rockford, IL).

Osteopontin- The full-length recombinant osteopontin was generated as a histidine-tagged protein (his-OPN) as previously described (Smith et al., 1996). Briefly, the full-length splice variant of human osteopontin (OP10) ref (Young et al., 1990) was cloned into pQE30 vector (Qiagen, Chatsworth, CA). Escherichia coli transformed with the his-OPN plasmid was grown in LB with 100 μg/ml ampicillin and induced with isopropyl-1-thio-β-

D-galactopyranoside for 4 hours. The His-OPN protein was purified from the bacterial cells according to the manufacturer's instructions. (QIAexpressionist kit, Qiagen).

Recombinant osteopontin N-terminal fragment was generated by thrombin cleavage of GST-30N fusion protein as described previously (Smith et al., 1996). The 30N OPN fragment was separated from the GST by cleaving with biotinylated-thrombin (Novagen, Madison, WI) (10 units/mg protein) at room temperature for 2 hours. Biotinylated-thrombin was then removed with UltraLink Immobilized NeutraAvidin (Pierce) and separated from the protein by centrifugation. Protein determinations were done using the Micro BCA Protein Assay Reagent Kit (Pierce).

Native osteopontin derived from the conditioned medium of aortic smooth muscle cell cultures and human urinary osteopontin were purified as previously described (Liaw et al., 1994). Briefly, human urine or conditioned media were dialyzed against PBS and fractionated over a DEAE-sepharose column with a linear salt gradient (0.15M-1M NaCl). Fractions containing osteopontin were absorbed to barium citrate and eluted with 0.2 mol/L sodium citrate The protein was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and it migrated at 70kd in a 12.5% gel.

Osteopontin Antibody- Anti-human osteopontin antibody (OP189) was produced in a goat immunized with 50 μg human urinary osteopontin. The osteopontin was purified as described above and mixed with Freund's adjuvant. Two booster injections,50 μg each, in incomplete Freund's adjuvant, were given 3 weeks apart after the initial immunization. Thirteen weeks after the initial immunization, serum was collected and the IgGs were purified by caprylic acid precipitation and separose column chromatography (Russo et al., 1983). The antibody was then dialyzed and stored frozen. Specificity was tested by western blot analysis (not shown) and enzyme-linked immunosorbant assay (see below)

HA and Osteopontin Binding Assay- The ability of CD44-Ig fusion proteins to bind hyaluronic acid and osteopontin was analyzed by ELISA. Maxisorp microtiter plates (Nunc Inc., Naperville, IL) were coated overnight at room temperature with human umbilical cord hyaluronic acid (Sigma Corp., St. Louis, MO) in 50 mM sodium bicarbonate buffer (pH 9.6) at a concentration of 10 μg/ml or the indicated amount of osteopontin in PBS. Wells were washed three times with PBS containing 0.05% Tween-

20 and blocked with 1x Specimen Diluent (Genetic Systems, Redmond, WA) for 1-2 hours at room temperature. Wells were then washed and incubated with CD44-Ig fusion proteins at various concentrations for 1 hour room temperature. After washing, wells were incubated with HRP-conjugated $(Fab')^2$ goat anti-human Ig gamma chain (Biosource, Camarillo, CA) at 1:5000 dilution in 1x specimen diluent for 1 hour at room temperature. After washing, bound HRP-antibody was detected using Chromagen-TMB diluted 1:100 in citrate buffered substrate (both from Genetic Systems). The absorbance was measured at wavelength 450 nm. To detect osteopontin on the wells, anti-osteopontin antibody (OP189) was added to wells followed by anti-goat HRP.

Cell Adhesion Assays- These assays were performed as described elsewhere (Liaw et al., 1994). Briefly, matrix proteins were coated onto 96-well Maxisorp microtiter plates (Nunc, Naperville, IL) overnight at 4°C and blocked one hour with PBS containing 10 mg/ml bovine serum albumin (BSA). Cells were resuspended in Dulbecco's medium containing 1 mg/ml BSA and preincubated with or without EDTA for 15 minutes at 37°C. WEHI-3B cells (100,000) were added to the wells and allowed to incubate for 45 minutes at 37°C. Attached cells were stained with toluidine blue, solubilized and quantitated by reading the absorbance at 595 nm. Under these conditions, absorbance was proportional to cell number (Liaw et al., 1994).

RESULTS

CD44-Ig Fusion proteins do not bind human recombinant osteopontin.

In a recent report, CD44 transfected cells expressing the CD44 v7-v10 variant was shown to interact with osteopontin. To determine if the standard form of CD44 or other variants of CD44 can interact with this protein, we examined the ability of CD44 receptor immunoglobulins (CD44-hIg) to directly bind osteopontin in an ELISA assay. Several different CD44 receptor immunoglobulin isoforms were available for use in this study. The CD44H-hIg is a chimeric protein that contains all the CD44 extracellular common exons, E1-E5, E15-E16, in frame with the hinge, CH2 and CH3 domains of a human IgG. CD44E-hIg is a similar construct which contains three additional exons (v8-v10). CD44 v3,v8-v10 and CD44 v3 contain the v3, v8-v10 and v3 respectively in addition to the

common exons. A schematic diagram of the CD44 splice variants used in these studies is shown in figure 4.1.

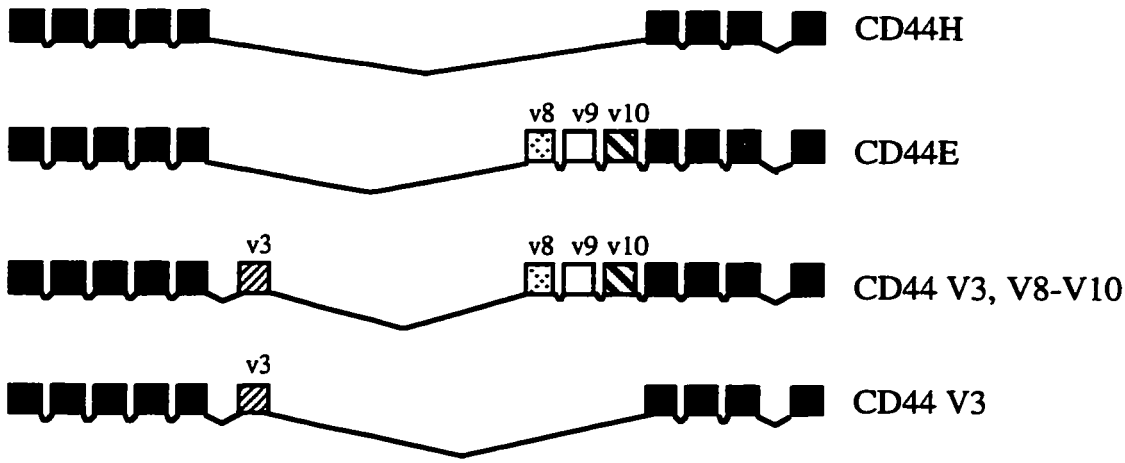

Figure 4.1. Schematic diagram of CD44-hIg variants used for ELISA. Four CD44-Ig constructs were used for these studies; CD44H-Ig, CD44E-Ig, CD44v3, v8-v10-Ig, and CD44 v3, v8-v10.

To measure CD44 interaction with OPN, soluble CD44-hIg fusion proteins (20 μg/ml) were incubated with 1 μg/ml osteopontin immobilized on 96 well plates. Bound CD44-hIg was detected by adding HRP-labeled anti-human IgG antibodies which recognize the Ig portion of the fusion protein. Hyaluronic acid, which was used as a control protein, supported binding of CD44H and CD44E and reduced binding to the CD44 v3 and CD44 v3, v8-v10 (figure 4.2A) as has been previouly shown (Bartolazzi et al., 1995; Bennett et al., 1995; Jackson et al., 1995). Reduced HA binding to CD44 variants containing v3 is due to glycosylation which inhibits the interaction with HA (Bartolazzi et al., 1996; Jackson et al., 1995). In addition, HA failed to bind a mutant CD44-hIg fusion protein that contains a point mutation in the extracellular domain (CD44HR41A) that has previously been shown to be important for HA interactions (Peach et al., 1993). Surprisingly, the CD44H form, as well as the CD44E, CD44 v3, v8-v10, and CD44 v3 all failed to bind recombinant human his-OPN. Similar results were also seen using 50 μg/ml CD44-hIg proteins (not shown) and 2 μg/ml, 10 μg/ml, and 20 μg/ml osteopontin. To rule out the

possibility that osteopontin was not successfully coated on the plate, goat anti-OPN antibodies were added in the place of CD44-hIg proteins to detect the OPN. The results demonstrate that there is significant binding of anti-OPN antibodies to OPN-coated, but not HA-coated wells indicating that the OPN coating is efficient (figure 4.2B).

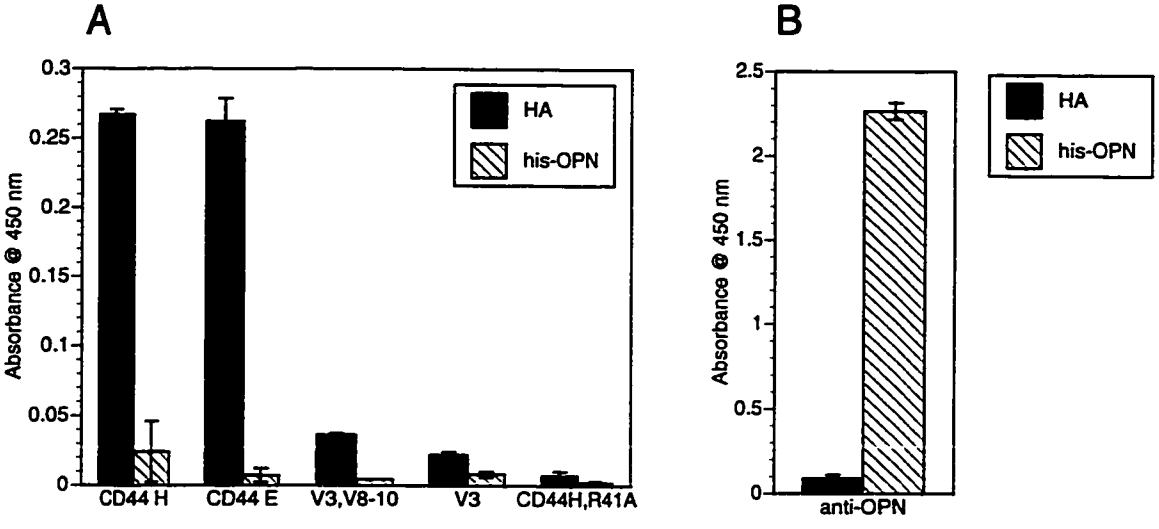

Figure 4.2. ELISA of CD44 interaction with human recombinant osteopontin and hyaluronic acid. A, CD44-hIg fusion proteins (20 ug/ml) were allowed to bind for 1 hour to wells coated with 10 ug/ml hyaluronic acid (HA) or 1 µg/ml human recombinant his-tagged osteopontin (his-OPN). The attached CD44-hIg proteins were detected by adding HRP-labeled anti-human IgG antibodies followed by substrate as described in "experimental procedures". B, anti-OPN antibodies were allowed to bind for 1 hour to wells coated with 10 ug/ml hyaluronic acid (HA) or 1 µg/ml human recombinant his-tagged osteopontin (his-OPN). The attached anti-osteopontin antibody was detected by adding HRP-labeled anti-goat IgG antibodies followed by substrate. Each data point represents the mean ± S.D. of triplicate samples.

CD44-HIG FUSION PROTEINS DO NOT BIND NATIVE OSTEOPONTIN OR RECOMBINANT OSTEOPONTIN FRAGMENTS.

Native osteopontin can be both heavily phosphorylated and glycosylated (Giachelli et al., 1995; Kasugai et al., 1991; Sorensen and Petersen, 1994; Sorensen and Petersen, 1995). In most cases, post-translational modification of OPN does not effect its function since recombinant OPN behaves similar to the native protein. There are however, several reports suggesting that glycoslyation and phosphorylation can be critical for some activities (Boskey et al., 1993; Ek Rylander et al., 1994; Hunter et al., 1994; Shanmugam et al.,

1997). To determine if post-translational modifications are important for CD44 interactions, we examined the ability of CD44-hIg proteins to bind several different native OPN preparations including human OPN purified from urine and rat OPN derived from pup rat SMCs (pup OPN). Smooth muscle cell derived OPN has previously been shown to be both glycoslyated and phosphorylated (Giachelli et al., 1995; Saavedra, 1994). In addition to native OPN, we also tested the N- and C- terminal recombinant osteopontin fragments expected to be formed following thrombin cleavage at the Arg169-Ser170 site. The N-terminal domain of OPN included amino acids 17-169 (10N and 30N). 30N and 10N fragments are identical except the 30N includes the alternative splice exon 5. Both of these fragments have previously been shown to support both $\alpha_v\beta_3$- and $\alpha_9\beta_1$-mediated adhesion. The C-terminal fragment (10C), contains amino acids 170-317. A function for this fragment has not yet been found. A schematic diagram of these proteins are shown in figure 4.3. In each experiment, HA was used as a control protein. The results clearly

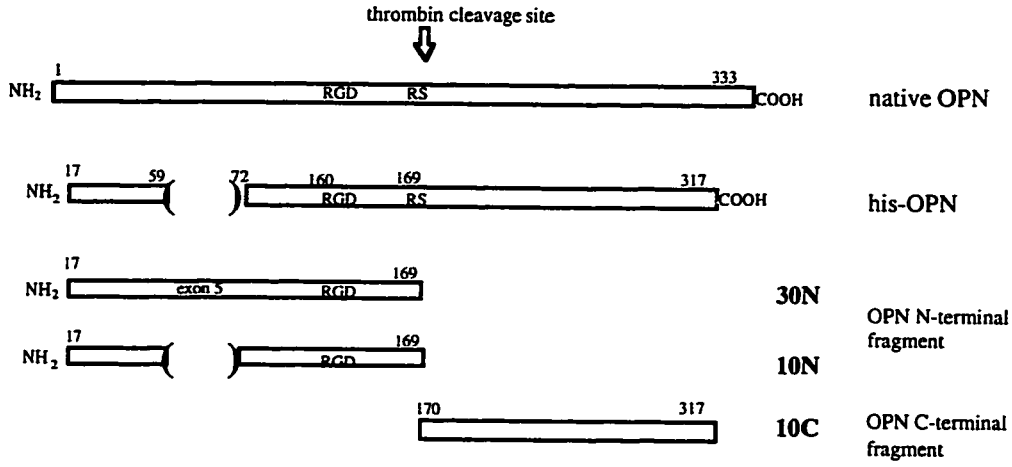

Figure 4.3. Schematic diagram of native osteopontin and human recombinant osteopontin fragments used for ELISA and adhesion assays. The native, full-length osteopontin preparations used in these studies was purified from rat pup smooth muscle cells and human urine. The full-length recombinant OPN was prepared as a His-tagged protein (His-OPN). All other osteopontin molecules were prepared as human recombinant GST-fusion proteins. The N- and C-terminal domains are fragments that are expected to be formed following thrombin cleavage. The C-terminal osteopontin domain (10C) contains amino acids 170-317. The N-terminal OPN fragments (30N and 10N) are alternatively spliced and include amino acids 17-169. the 30N splice variant contains an additional 14 amino acids (NAVSSEETNDFKQE), which correspond to exon 5.

show that CD44-hIg does not interact with any of the osteopontin preparations tested (figure 4.4). All forms of osteopontin were sufficiently coated onto the wells as detected by anti-OPN antibodies (not shown). In addition all preparations of OPN except for C-OPN, were found to be biologically active by adhesion assays. The C-OPN recombinant fragment which doesn't contain the RGD site fails to interact with any of the cell lines we have tested and a function for this fragment has not yet been identified.

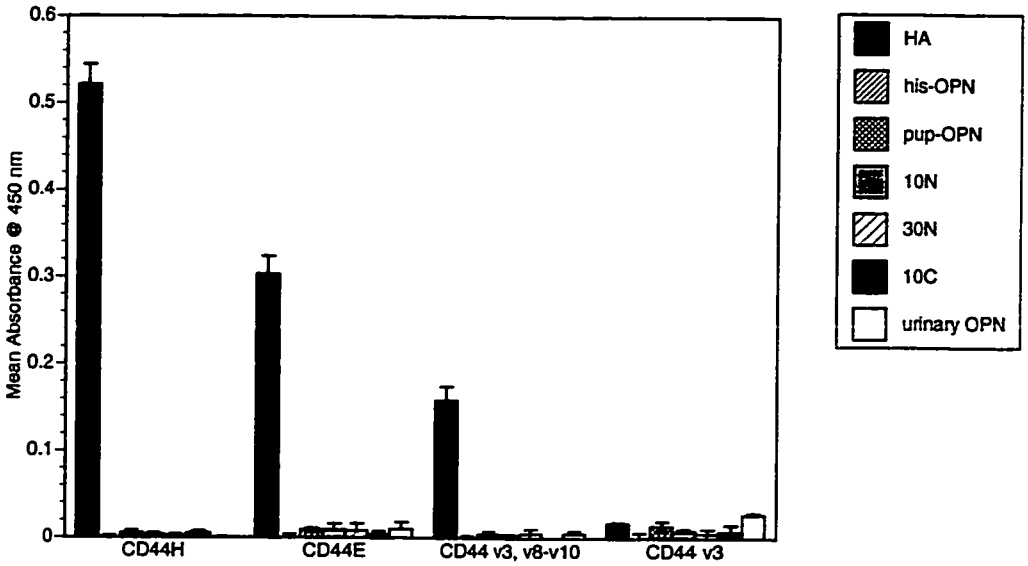

Figure 4.4. ELISA of CD44 interaction with native osteopontin and recombinant osteopontin fragments. CD44-hIg fusion proteins (20 µg/ml) were allowed to bind for 1 hour to wells coated with 10 µg/ml hyaluronic acid (HA),5 µg/ml human recombinant his-tagged osteopontin (his-OPN), 5 µg/ml native rat pup smooth muscle-derived osteopontin (pup-OPN), 5 µg/ml human urinary ostopontin, and 5 µg/ml recombinant osteopontin fragments (30N, 10N, and 10C). The attached CD44-hIg proteins were detected by adding HRP-labeled anti-human IgG antibodies followed by substrate as described in "experimental procedures". Each data point represents the mean ± S.D. of triplicate samples.

DIVALENT CATIONS ARE REQUIRED FOR ADHESION OF WEHI-3B CELLS TO OSTEOPONTIN.

The monocytic line, WEHI-3B, was shown by Weber et al (Weber et al., 1996) to bind osteopontin in a ligand binding assay. To determine if WEHI-3B can interact with plate-bound osteopontin, we performed cell attachment assays with full-length recombinant osteopontin. A small amount of WEHI-3B adhesion to osteopontin was seen (figure 4.5), however, adhesion was completely inhibited in the presence of EDTA. Since divalent

cations are necessary for integrin binding, the inhibition by EDTA suggest adhesion of WEHI-3B to plate-bound OPN is mediated through integrin receptors rather then CD44.

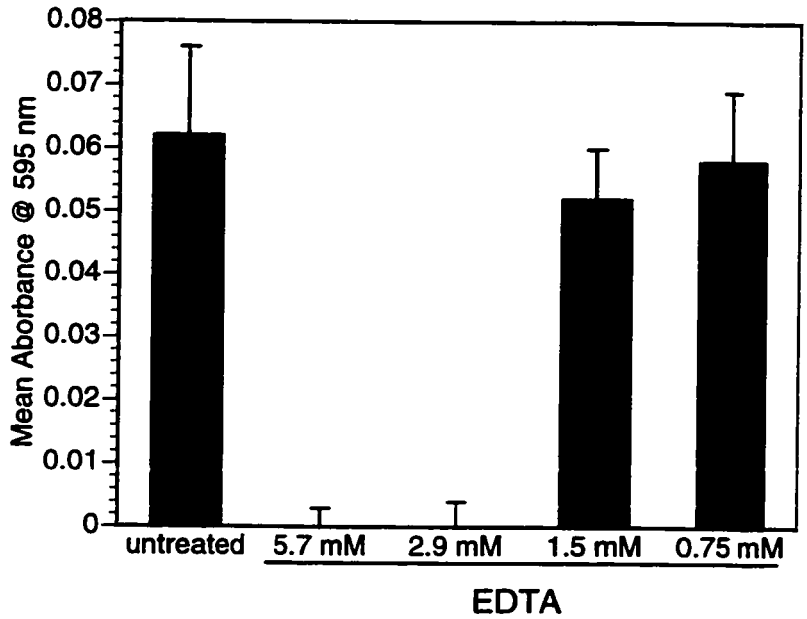

Figure 4.5. Adhesion of WEHI-3B cells to osteopontin in the presence of EDTA. WEHI-3B cells were preincubated with and without EDTA at the indicated concentrations for 15 minutes at 37°C before plating on wells coated with 100 nM human recombinant osteopontin. The attached cells were fixed and stained with toluidine blue as described under "Experimental Procedures". Each data point represents the mean ± S.D. of triplicate samples. Nonspecific cell adhesion as measured on BSA-coated wells was subtracted.

DISCUSSION

This study examined the potential interaction of CD44 with osteopontin. We used soluble CD44-hIg fusion proteins to analyze the interaction of several different CD44 splice variants to OPN by ELISA. The results demonstrate that the CD44 variants used in this study do not interact with several native and recombinant forms of osteopontin.

Using four different splice variant CD44-hIg fusion proteins, we have shown that CD44H, CD44E, CD44 v3,v8-v10 and CD44 v3 could not interact with human recombinant his-tagged OPN. Further analysis demonstrated that several additional preparations of osteopontin, including recombinant fragments of human OPN formed following thrombin cleavage, native OPN derived from rat SMCs and native OPN derived from human urine, could also not support the binding of any CD44 isoforms used. These results are contradictory to those published by Weber et al, that showed a CD44 transfected cell line could mediate adhesion and migration to OPN (Weber et al., 1996).

One explanation for the lack of CD44-mediated attachment to OPN in this study is that the interaction may depend on a particular CD44 splice variant. Specific isoforms has previously been shown to be important for the binding of CD44 to other ligands. For example, the binding of CD44 to HA is dependent on the glycosylation state, which varies according to the alternative splice variant expressed (Bartolazzi et al., 1996; Bennett et al., 1995; Katoh et al., 1995; Lesley et al., 1995). In addition, the ability of CD44 to bind and present heparin-binding growth factors is dependent on the v3-containing isoforms (Bennett et al., 1995). In the study by Weber et al, cells had been trasfected with a CD44 isoform derived from an osteosarcoma cell line which contains v7-v10. For our study, we used four different CD44 variants. CD44H is the most common form and is found on hemopoietic and mesoderm cells (Haynes et al., 1989; Stamenkovic et al., 1989). This variant was also upregulated in vascular tissue following balloon injury The other variants include CD44E, CD44v3, v8-v10, and CD44v3. CD44E is the epithelial form of CD44. CD44 variants that contain v3 have been shown to bind heparin binding growth factors through the heparin sulfate side chain (Bennett et al., 1995). CD44 v7-v10 was not available for use in these studies.

In addition to the interaction of OPN with CD44v7-v10 expressing transfectants, Weber et al also demonstrated a CD44-specific attachment of a monocytic cell line, WEHI-3B, to OPN. It is unclear from that study what particular splice variant(s) are expressed by these cells. In our own experiments, we found that WEHI-3B could attach to recombinant human his-OPN, however addition of 2.3 mM EDTA eliminated all adhesion suggesting that the interaction is mediated through integrin receptors or other calcium-dependent receptors rather then CD44. One possible explanation for the discrepancy between these two studies, is that a particular source of OPN is required for WEHI-3B binding.

Osteopontin has a number of post-translational modifications including phosphorlyation and glycosylation. In most cases, these post-translational modifications do not alter its biological activity, since bacterial expressed recombinant proteins behave in a similar manner to native OPN purified from several different sources (Liaw et al., 1995; Xuan et al., 1994). There are however, several reports where post-translational modifications have been shown to alter OPN function. For example, sialylation of OPN is crucial for receptor-mediated binding to tsB77 cells. In addition, the phosphorylation state of OPN affects its interaction with osteoclasts (Ek Rylander et al., 1994) and normal rat kidney cells (Singh et al., 1990), as well as its ability to inhibit hydroxyapatite formation (Boskey et al., 1993; Hunter et al., 1994). In the studies described in this paper, we used recombinant OPN and native OPN previously shown to be phosphorlyated and glycosylated (Giachelli et al., 1995). All preparations failed to interact with CD44. We can not however, rule out the possibility that a post-translationally modified form of OPN not used in this study could interact with CD44.

In conclusion, we have found that the standard CD44 and three CD44 splice variants do not interact with several different osteopontin preparations. These studies suggest that CD44-OPN interactions may not be a common event in vivo, and may be limited to a specific CD44 splice variant(s), and/or a particular modified form of osteopontin.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Osteopontin is a multifunctional protein with a number of interesting structural domains. Studies of other adhesive proteins with similar multidomain structures indicate that the ability to bind various molecules such as integrins, proteases, growth factors, and matrix proteins through distinct regions probably explains many of their multifunctional properties. A number of studies have also indicated that some functional domains harbor biological activities not detected within the intact molecules. (Homandberg et al., 1986; Homandberg et al., 1992; Horton et al., 1994; Humphries and Ayad, 1983; Montgomery et al., 1994; Werb et al., 1989). These cryptic activities may be exposed following conformational changes, or may be liberated by proteolytic fragmentation of the intact proteins. Proteolytic cleavage of proteins within the extracellular matrix is particularly important in remodeling tissues where proteases and adhesive proteins are often upregulated and likely co-localize.

The studies described in this dissertation were designed to investigate: 1) The function of particular structural domains of osteopontin and 2) the biological consequences of protease-osteopontin interactions. Specifically, these studies focused on the regulation of osteopontin function by the protease thrombin, and include: 1) the identification of receptors which interact with thrombin-cleaved osteopontin fragments and the consequence of these interactions, 2) the structural domains important for the functional properties of the osteopontin fragment and 3) the interaction of osteopontin and osteopontin fragments with the non-integrin receptor, CD44. These studies support the hypothesis that proteolytic cleavage of osteopontin generates biologically functional fragments with distinct properties from the intact molecule and that osteopontin fragments may be important components of the remodeling vasculature. The following conclusions can be drawn from this work:

1. Osteopontin adhesive interactions can be regulated by proteolytic fragmentation. Following thrombin cleavage, the N-terminal osteopontin fragment contains two distinct integrin binding activities. One is an enhanced $\alpha_v\beta_3$-binding activity compared to the intact protein. This interaction is RGD-dependent. The second is a cryptic binding activity mediated through the $\alpha_9\beta_1$ integrin receptor: a function not found in the full-length molecule. These findings are significant in that both osteopontin and proteolytic enzymes are often found co-localized during inflammation and tissue remodeling. The functional properties of the thrombin-cleaved osteopontin protein suggests that as the remodeling matrix is exposed to proteases, cellular interactions may be altered.

2. The RGD domain is critical for $\alpha_9\beta_1$-mediated adhesion and migration to the N-terminal domain of osteopontin. Since the $\alpha_9\beta_1$ integrin recognizes the N-terminal osteopontin fragment and not the full-length molecule, these data supports the idea that the RGD domain in osteopontin exists in a thrombin sensitive conformation. These observations also suggest that $\alpha_9\beta_1$ has specific requirements in regards to its recognition of an RGD site. Potentially this finding could be exploited to generate specific antagonists which block $\alpha_9\beta_1$.

3. Osteopontin-CD44 interactions may not be a common occurrence *in vivo* and may be limited to specific CD44 isoform(s) and/or particular modified form of osteopontin. These results emphasize the importance of knowing what receptor variants are expressed on the cell types or tissues of interest to make more accurate predictions of potential receptor/ligand interactions.

The interaction of cells with extracellular matrix proteins plays an important role in a variety of biological processes, such as embryonic development, inflammation, and tissue remodeling. Osteopontin has been implicated in a number of diseases associated with remodeling including atherosclerosis and restenosis. Localization studies showing osteopontin in human atherosclerotic plaque, restenotic lesions and rat arterial injury models all implicate a role for osteopontin in vascular remodeling. The use of neutralizing monoclonal antibodies in the rat balloon injury model confirmed its importance in vascular repair. In addition, osteopontin can mediate the migration and adhesion of smooth muscle cells and endothelial cells, two processes important in vascular diseases.

Although osteopontin contains a cleavage site for thrombin, very little is known about how proteolytic fragmentation of osteopontin effects its function. An understanding of how proteases regulate osteopontin interactions with the cell will be important in recognizing the role osteopontin plays in remodeling tissues and vascular pathologies. To address this issue, we investigated the biological function of thrombin-cleaved osteopontin fragments.

To determine if proteolytic fragments of osteopontin are functional, we tested the ability of thrombin-cleaved osteopontin to support cell adhesion and migration. We observed that the N-terminal osteopontin fragment following thrombin cleavage is not only functional, but has additional and enhanced properties not found in the full-length molecule: The N-

terminal osteopontin fragment enhanced cell adhesion mediated through the $\alpha_v\beta_3$ integrin and contained a cryptic $\alpha_9\beta_1$ cell binding activity not seen in the full length molecule.

Interactions with Thrombin-Cleaved Osteopontin through the $\alpha_v\beta_3$ integrin

We have observed that the N-terminal osteopontin fragment has enhanced $\alpha_v\beta_3$-mediated activity compared to native osteopontin. This data is consistent with studies by Senger et al, who showed that thrombin cleavage of osteopontin enhanced the adhesion and migration of a number of cell lines including microvascular endothelial cells (Senger et al., 1996). These findings are significant in terms of the potential role for osteopontin-$\alpha_v\beta_3$ interactions in vascular remodeling. Both $\alpha_v\beta_3$ and osteopontin are associated with smooth muscle cells and endothelial cells in atherosclerotic plaques (Giachelli et al., 1993; Hoshiga et al., 1995). In a complicated atherosclerotic plaque, episodes of intraplaque hemorrhage or plaque disruption with thrombosis may promote osteopontin cleavage and enhancement of osteopontin's adhesive and migratory properties. Enhanced smooth muscle cells and endothelial cell migration could facilitate reendothelialization and the remodeling of a newly formed thrombus, thereby contributing to plaque growth.

Integrin $\alpha_v\beta_3$ interactions with the N-terminal domain of osteopontin may also be important in the process of angiogenesis, since both molecules, osteopontin and $\alpha_v\beta_3$, are expressed with microvessels in the adventia and the plaque (Giachelli et al., 1993; Hoshiga et al., 1995). Angiogenesis is associated with microvascular permeability resulting in the extravasation of plasma proteins (Dvorak et al., 1995; Senger et al., 1996). A consequence of extravasated coagulation factors may be the cleavage of osteopontin by thrombin in these newly formed vessels and could potentially regulate endothelial cell migratory and/or adhesive interactions during angiogenesis. Figure 5.1 shows a model of how osteopontin-protease interactions may regulate angiogenesis. Several studies support this hypothesis. 1) Senger et al showed that co-injection of osteopontin together with VPE/VEGF, which promotes vascular permeability, resulted in rapid osteopontin cleavage by endogenous thrombin (Senger et al., 1996), 2) thrombin cleaved osteopontin enhanced endothelial cell migration *in vitro* (Senger et al., 1996), 3) antibodies to $\alpha_v\beta_3$, the receptor for both osteopontin and thrombin-cleaved osteopontin, block tumor angiogenesis *in vivo* by inducing apoptosis of angiogenic blood vessels (Brooks et al., 1994; Drake et al., 1995).

and 4) osteopontin/$\alpha_v\beta_3$ interactions mediate endothelial survival *in vitro* (Scatena, 1997). Together, these data suggests that thrombin-cleavage of osteopontin could help facilitate the $\alpha_v\beta_3$-mediated processes critical for angiogenesis including endothelial cell survival and migration.

Cryptic $\alpha_9\beta_1$ Binding Activity

An exciting observation that came out of this study, was that the N-terminal osteopontin fragment could interact with $\alpha_9\beta_1$: an integrin that had not been previously identified as an osteopontin receptor. Interestingly, only the N-terminal osteopontin fragment and not the intact molecule could mediate $\alpha_9\beta_1$-dependent cell adhesion and migration, suggesting that a cryptic domain exists in the full-length protein. These data also demonstrates that in addition to its place in the coagulation cascade, thrombin can regulate osteopontin receptor specificity. Although the biological significance of this regulatory function *in vivo* is not yet known, it is interesting to note that both $\alpha_9\beta_1$ and osteopontin are often found in developing diseased tissues. Osteopontin is highly upregulated at sites of inflammation, tissue remodeling, and in areas surrounding tumors. These are also sites were thrombin and thrombin-cleaved osteopontin fragments are likely to be found. The $\alpha_9\beta_1$ integrin has also been shown to be highly expressed in remodeling tissue. It is upregulated during epithelial regeneration following debridement in the cornea (Stepp and Zhu, 1997) and amyloid light-chain amyloidosis (Turbat Herrera et al., 1997). In the developing murine embryo, α_9 is detected late during gestation in visceral and vascular smooth muscles, squamous epithelia, and epithelium of the choroid plexus and skeletal muscle. The α_9 expression in the vascular smooth muscle is limited to E12.5-16.5 suggesting it may play a role in vasculogenesis (Wang et al., 1995). Importantly, both osteopontin and α_9 were shown to be expressed in the ductus arteriousus during the vascular remodeling process required for ductus closure (Clyman et al., 1996). Although co-localization studies have not yet been done, it is possible that both $\alpha_9\beta_1$ and osteopontin will be co-expressed in some remodeling tissues where proteases are likely to be found. Preliminary results suggest that $\alpha_9\beta_1$ integrin can be located by western blotting in diseased vascular tissue. If further studies demonstrate that $\alpha_9\beta_1$ is co-localized with osteopontin, one could speculate that osteopontin-protease interactions may initiate adhesion or migration of cells which

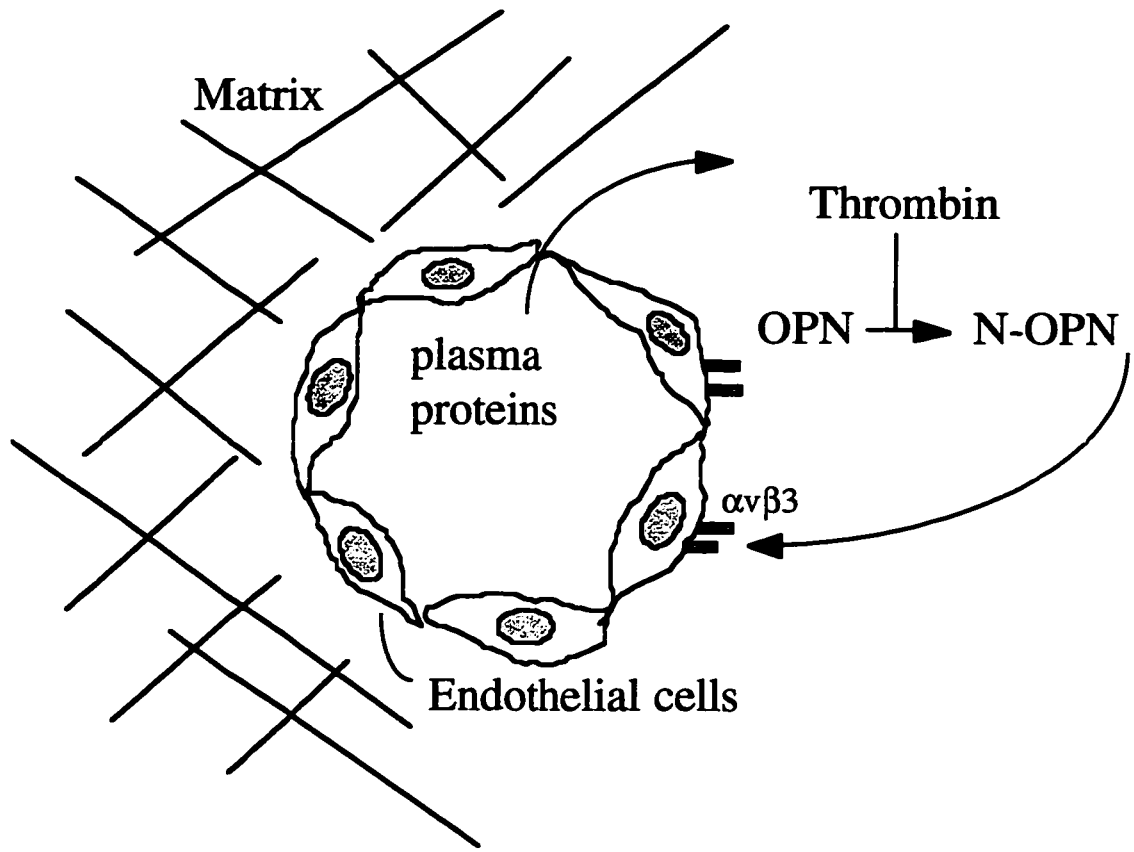

Figure 5.1. Hypothetical model for osteopontin's role in angiogenesis. Based on the data listed above, the following hypothetical model for how osteopontin and its proteolytic fragments regulate angiogenesis: Mircovascular permeability associated with angiogenic vessels results in the extravasation of plasma proteins and the cleavage of osteopontin. The N-terminal osteopontin fragment interacts with the $\alpha_v\beta_3$ receptor to mediate enhanced adhesion, migration and survival the angiogenic endothelial cells: three processes important for the angiogenic process. The full-length osteopontin could simultaneously support the same functions but to a lesser degree.

normally would not bind osteopontin. Alternatively, this new interaction could initiate a signaling cascade leading to gene expression important for the remodeling process.

$\alpha_9\beta_1$ recognition site on the N-terminal osteopontin fragment

An additional observation made in these studies was that $\alpha_9\beta_1$ mediated adhesion and migration to the N-terminal osteopontin fragment was dependent on the RGD sequence. This finding was initially unexpected since tenascin, the only other known ligand for $\alpha_9\beta_1$, mediates adhesive interactions through a non-RGD domain. However, a direct comparison of tenascin and osteopontin, with either mutant or wildtype RGD domains, confirmed that these two molecules interact with $\alpha_9\beta_1$ through distinct mechanisms.

The results of these experiments do not explain why thrombin cleaved osteopontin fragments and not the full-length molecule interact with $\alpha_9\beta_1$. The RGD site is exposed in both native osteopontin and the osteopontin fragment, yet $\alpha_9\beta_1$ only interacts with the osteopontin fragment. One possible explanation for this observation is that sequences exposed following thrombin cleavage are necessary for $\alpha_9\beta_1$-RGD interactions. For example, fibronectin contains a pentapeptide site that synergistically enhances the cell-adhesive activity of the fibronectin-RGD sequence (Aota et al., 1994). It is possible that the N-terminal osteopontin fragment may contain a synergy site that is unavailable in the full-length molecule, which acts to stabilization of $\alpha_9\beta_1$-RGD interactions. We pursued studies which targeted specific sequences in the osteopontin N-terminal domain that might facilitate $\alpha_9\beta_1$-RGD interactions. However, these studies did not identify any additional domains important for the interaction.

An alternative explanation for why the RGD domain in the full-length molecule is not recognized by $\alpha_9\beta_1$ is that the RGD site takes on a distinct conformation following thrombin cleavage. Integrin $\alpha_9\beta_1$ could be particularly sensitive to these changes in RGD presentation. The RGD site in osteopontin is thought to be in a loop flanked by β-sheets on either side (Denhardt and Guo, 1993). Since the thrombin cleavage site is just 6 amino acids away from this site, it is possible that the β-sheet on the C-terminal end has been disrupted making the RGD site more flexible and thus accessible to $\alpha_9\beta_1$ integrin. For example, structural analysis of the RGD site in fibronectin shows that it exists in a loop

bound by β-strands (Dickinson et al., 1994; Main et al., 1992), However, the structure for the tenth fibronectin III repeat is too flexible to determine suggesting that the RGD is rigid in the larger fragment and more flexible in the smaller form. The model in figure 5.2 explains how the cleavage of osteopontin could lead to changes in RGD conformation. The conformational change would have two consequences: exposing a $\alpha_9\beta_1$ binding conformation not normally present in the full-length protein, and enhancing $\alpha_v\beta_3$-cell interactions.

The search for antagonists with high specificity for individual integrin receptor sites is a major focus of the pharmaceutical industry. Use of RGD peptides or compounds designed to mimic RGD have been used in various applications *in vivo*. For example, RGD peptides that inhibit the function of $\alpha_{IIb}\beta_3$ integrin are now in clinical trials as anti-thrombotics. Peptides that target $\alpha_v\beta_3$ are being tested as treatments for osteoporosis and tumour angiognesis based on their ability to: 1) inhibit the interaction of osteoclasts with bone and prevent bone degradation *in vitro* and *in vivo* (Engleman et al., 1997; Fisher et al., 1993) and 2) inhibit the survival of endothelial cells undergoing angiogenesis. Understanding the differences between the distinct RGD conformations in the intact osteopontin molecule and the osteopontin fragment may be important in the design of small peptide antagonists which affect $\alpha_9\beta_1$ preferentially over $\alpha_v\beta_3$ or other α_v-containing osteopontin integrin receptors.

In the process of identifying RGD as the functional domain for $\alpha_9\beta_1$, we discovered that the $\alpha_9\beta_1$ integrins appears to be more promiscuous then the α_v receptors in its ability to recognize the RGD site. Mutation of the RGD site to RGE, completely inhibited all α_v-mediated functions. However, the same mutation still allows 50% functional activity with the $\alpha_9\beta_1$ receptor. These result may be important in the design of peptide antagonists with the opposite function as those described above, ie. antagonists that specifically inhibit α_v-mediated functions preferentially over $\alpha_9\beta_1$ functions.

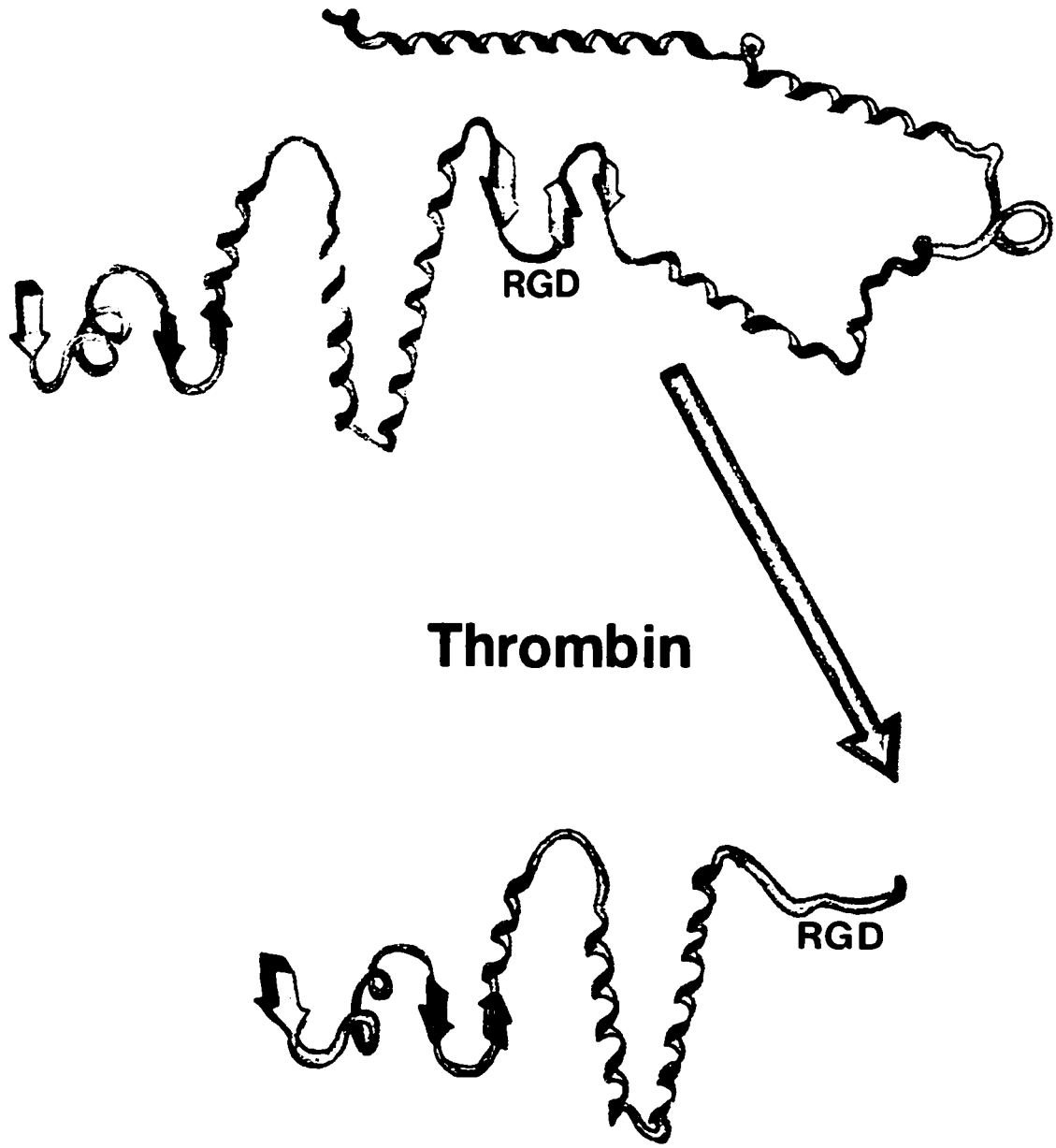

Figure 5.2. Model for structural changes in osteopontin induced by thrombin cleavage. The full-length osteopontin molecule exists in a loop bound by β sheets. This conformation would support α_v-, but not $\alpha_9\beta_1$-mediated interactions. Following thrombin cleavage, the restrained nature of the RGD may be lost and the conformation of RGD changed. The change in the structure surrounding the RGD would expose a $\alpha_9\beta_1$ binding conformation and enhance $\alpha_v\beta_3$ interactions.

Adhesive Interactions between CD44 and Osteopontin

The finding by Weber et al (Weber et al., 1996), that osteopontin is a ligand for the non-integrin receptor, CD44 was initially very exciting. CD44-osteopontin interactions could potentially be very important in diseases of the vasculature because both molecules are highly expressed in remodeling vascular tissue and in angiogenic vessels. Both molecules have also been implicated in smooth muscle cell adhesive and migratory functions. Weber et al demonstrated that CD44 mediated cellular adhesion and migration to osteopontin (Weber et al., 1996), and that the activity was found in the C-terminal osteopontin fragment (Weber et al., 1997). Presently, this is the first report suggesting a function for the C-terminal domain. The C-terminal fragment lacks the RGD domain, but contains amino acid sequences with heparin binding and calcium binding homology. The heparin binding domain is of particular interest because some of the CD44 splice variants are modified with heparin sulfate, suggesting a possible mechanism for attachment. However, the studies presented in this dissertation suggests that osteopontin-CD44 interactions are not common. The most widely expressed CD44 isoform, CD44H, which can be found in remodeling vascular tissue and angiogenic vessels, failed to interact with osteopontin. Other splice variants, often associated with tumor metastasis, also failed to interact with osteopontin. Although CD44-osteopontin interactions may not be common, it is possible that splice variants or modified forms of osteopontin could be particularly important. In our study, several osteopontin preparations were used, including native osteopontin previously shown to be both phosphorylated and glycosylated, suggesting that if post-translational modifications are important, it must be very specific. These data presented here are important in that it demonstrates osteopontin-CD44 interactions may not be a common event and may be limited to a very specific CD44 splice variant(s), and/or a particular modified form of osteopontin. Until it is clear which form(s) are important, it will be difficult to speculate how this interaction may be relevant *in vivo*.

One hypothesis forming the basis of these studies was that proteolytic cleavage of osteopontin generates functional fragments important in vascular disease. We have determined that proteolytic fragments are not only biologically functional, but contain additional activities not associated with the full-length molecule. Whether or not these fragments are generated and are biologically significant *in vivo* still needs to be addressed. Continuous production of proteases in chronic non-healing pathologies, could induce high

local concentrations of osteopontin fragment. In attempt to identify proteolytic fragments *in vivo*, monoclonal antibodies are now in process of being made that are designed to recognize the N-terminal osteopontin fragment, but not the full-length molecule. When these antibodies become available, localizing osteopontin fragments *in vivo* can be carried out.

Future directions-proposal

It is not clear as to what extent extracellular matrix molecules and the protease cascade systems are crucial *in vivo*. A number of issues still need to be explored before the significance of osteopontin-$\alpha_9\beta_1$ interactions can be proposed. 1) Are $\alpha_9\beta_1$ integrin and osteopontin fragments co-localized in remodeling tissues? 2) What is the importance of the regulatory function of osteopontin cleavage by thrombin or other proteases in vascular cells? 3) Are there additional mechanisms, other then proteolytic cleavage, in which $\alpha_9\beta_1$-binding site is exposed? Addressing these issues may lead to new perspectives about the role of protease-integrin-extracellular matrix systems.

1) Are $\alpha_9\beta_1$ integrin and osteopontin fragments co-localized in remodeling tissues?

The key to understanding if $\alpha_9\beta_1$-osteopontin interactions are important *in vivo*, is determining if the two molecules are co-expressed in the tissues of interest. A polyclonal antibody directed against the human $\alpha_9\beta_1$ integrin has previously been developed. However, due to availability and problems associated with its use in immunocytochemistry, it has not been possible to carry out localization studies of the α_9 integrin in the vasculature. In an effort to determine if $\alpha_9\beta_1$ is upregulated in remodeling tissues, an α_9 antibody is being produced in our laboratory using a peptide from the C-terminal end of the N-terminal domain of osteopontin, which contains the RGD, as an antigen.

To determine if osteopontin fragments are co-localized to remodeling tissues. Monoclonal antibodies directed against the N-terminal osteopontin fragment, but not the intact molecule are in the process of being screened. The antigen used for immunizations was the peptide described above conjugated to bovine serum albumin (DTYDGRGDSVVYGLR). This

peptide sequence was chosen because of the presumed conformational changes around this site in the intact and N-terminal fragment. The hybridomas were screened with the N-terminal fragment. Any positive clones will then be screened for selective recognition of the N-terminal fragment and not the full-length molecule. Any positive antibodies will then be analyzed further for its ability to recognize native osteopontin fragments by ELISA, western blot, and immunocytochemistry.

When anti-N-terminal osteopontin and α_9 antibodies are made, they will be used in immunocytochemistry experiments to determine if these two molecules co-localize. Tissues of interest include: rat carotid following vascular injury, human atherosclerotic plaque and restenotic tissue. In addition, osteopontin has been implicated in a number of other diseases associated with remodeling. It would be interesting to explore the possible interactions of α_9 with osteopontin in cancer and kidney disease.

2) What is the importance of the regulatory function osteopontin cleavage by thrombin in the vasculature?

If is becomes clear that α_9 is upregulated in the vasculature, studies will be aimed at investigating *in vitro* consequences of α_9-osteopontin interactions. Unfortunately the α_9 integrin is rapidly down regulated in most cultured cells, therefore to pursue these studies, the α_9 integrin will need to first be transfected into cell types of interest. For example, if it is clear that smooth muscle cells express α_9 following vascular injury, cultured smooth muscle cells will be transfected with α_9 and used *in vitro* functional assays. Since α_9 has been show to mediate both adhesion and migration to the N-terminal domain of osteopontin, experiments will be done to determine if, like melanoma cells, smooth muscle cells can participate in these functions. The significance of $\alpha_9\beta_1$-interactions with osteopontin can also be assessed *in vivo* by using neutralizing α_9 or anti-N-terminal osteopontin antibodies in the rat balloon injury model.

3) Are there additional mechanisms, other then proteolytic cleavage, in which $\alpha_9\beta_1$-binding site is exposed?

In the studies presented in this dissertation, it was shown that osteopontin contains a cryptic $\alpha_9\beta_1$ binding site exposed following thrombin cleavage. Interestingly, the $\alpha_9\beta_1$

binding site is the RGD domain, which is also exposed in the full-length molecule. The RGD site in the intact protein is accessible to $\alpha_v\beta_3$, but not $\alpha_9\beta_1$ integrin. We have speculated that conformational changes, induced following thrombin cleavage, allows $\alpha_9\beta_1$ interactions. Is it possible that this same conformational change can be exposed in the intact molecule by surface adsorption or interaction with heparin, hydroxyapatite or Ca^{++}? For example, studies of the fibronectin molecule have demonstrated that the amino-terminal domain of the fibronectin molecule binds to heparin domain with high affinity and that internal associations between net negative and positive charged domains may lead more compact folding of the molecule (Erickson and Carrell, 1983). If similar electrostatic associations occurred in osteopontin between the positive charged heparin binding site and the negative charged Ca^{++} binding domain, the association of heparin or Ca^{++} would disrupt the ionic interaction and potentially alter the conformation of the intact osteopontin molecule.

In an initial attempt to determine if heparin binding exposes $\alpha_9\beta_1$ adhesive capabilities, different concentrations of heparin and osteopontin mixtures will be coated on 96 well plates and their ability to support $\alpha_9\beta_1$-mediated adhesion of Mo melanoma cells measured. In addition, the gain of anti-N-terminal osteopontin antibody binding (produced in aim 1) can be used to monitor conformational changes. In wells containing only osteopontin, we would expect to not see any $\alpha_9\beta_1$-mediated adhesion or antibody binding. If conformational changes due to heparin binding exposes $\alpha_9\beta_1$ adhesive activities, an increase in Mo adhesion and antibody binding should be seen when heparin is coated with osteopontin. To determine if interactions between opposite charged domains such as the heparin binding domain and the Ca^{++} binding domain are responsible for keeping the RGD conformation cryptic, heparin domain peptides or aspartic acid rich peptides will be added to osteopontin prior to coating 96 well plates. If an ionic association is taking place between the two domains, the peptides should interfere and promote the exposure of $\alpha_9\beta_1$ binding site. To confirm this interaction, the affinity of N-terminal domain and Ca^{++} binding domain can be measure by affinity binding assays. In addition, circular dichroism analysis can be done to measure ionic strength-dependent changes of osteopontin structure to provide further evidence that disruption of ionic interaction alters osteopontin conformation.

Accessibility of the $\alpha_9\beta_1$-binding site may also be affected by molecular changes induced by interactions with extracellular ions. For example, Ca^{++} binding to thrombospondin preserves thrombospondin in an adhesive conformation (Sun et al., 1992). Functional changes that might be induced by Ca^{++} binding is particularly relevant to osteopontin because this protein contains an aspartic rich sequence which can bind large amounts of calcium and has been implicated in mineralization and dystrophic calcification. To look for accessibility of the $\alpha_9\beta_1$ adhesive capabilities in the presence of extracellular ions, osteopontin will be coated on to 96 well plates with increasing doses of Ca^{++} and the ability of osteopontin to support $\alpha_9\beta_1$-mediated adhesion monitored by Mo cell adhesion. If a difference in $\alpha_9\beta_1$-mediated adhesion is observed, these experiments could be complimented by structural analysis such as circular dichroism analysis or solution scattering to determine shape changes with increasing concentrations of Ca^{++}.

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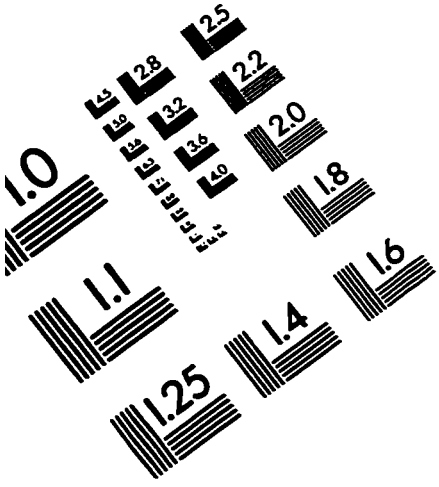
BOOK CHAPTERS:

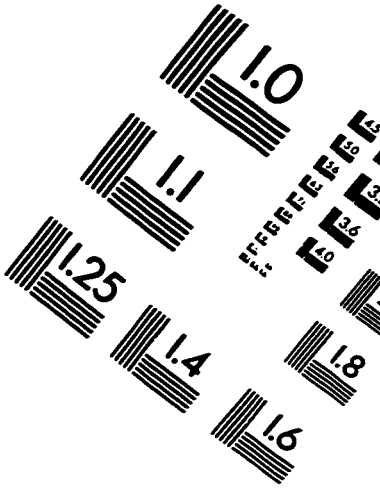
Giachelli C.M. and Smith, L.L. Role of Osteopontin in Health and Diseases. In Cell Adhesion Molecules and Matrix Bioscience, Editor, S. A. Mousa, (in press) 1997

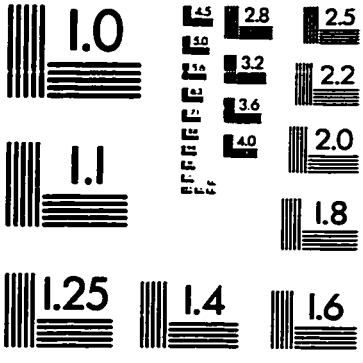
IN PREPARATION:

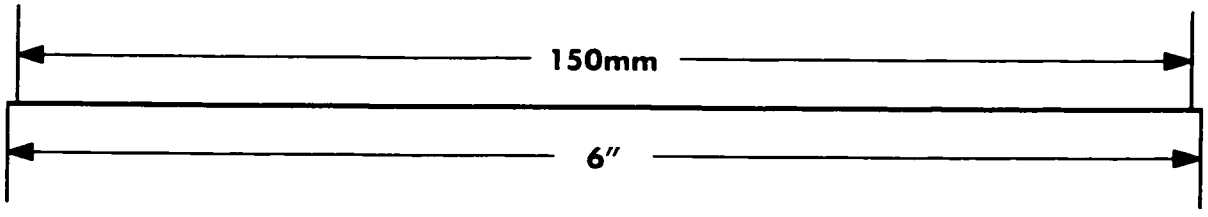
Smith, L.L., Greenfield, B., Aruffo, S. and Giachelli, C.M. Osteopontin does not interact with soluble CD44-hIg protein and several CD44 splice variants.

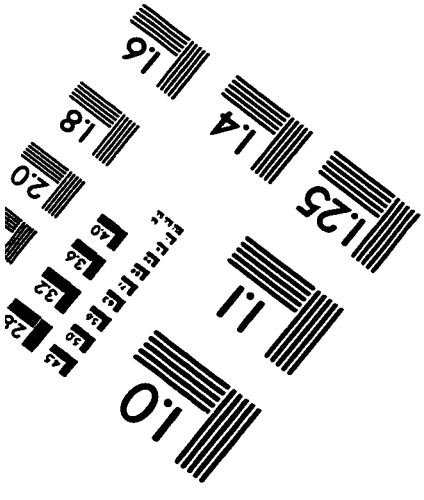
IMAGE EVALUATION TEST TARGET (QA-3)

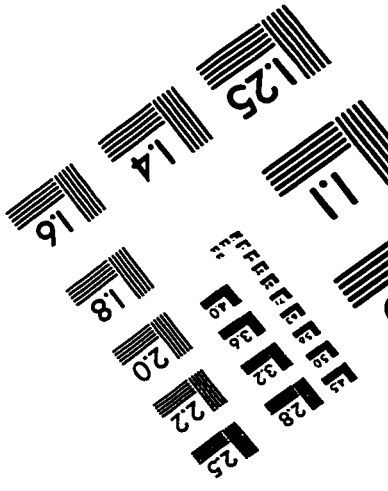












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