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Responses of Vascular Smooth Muscle Cell to Extracellular Matrix Degradation

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Abstract Vessels remodel to compensate for increases in blood flow/pressure. The chronic exposure of blood vessels to increased flow and circulatory redox-homocysteine may injure vascular endothelium and disrupt elastic laminae. In order to understand the role of extracellular matrix (ECM) degradation in vascular structure and function, we isolated human vascular smooth muscle cells (VSMC) from normal and injured coronary arteries. The apparently normal vessels were isolated from explanted human hearts. The vessels were injured by inserting a blade into the lumen of the vessel, which damages the inner elastic laminae in the vessel wall and polarizes the VSMC by producing a pseudopodial phenotypic shift in VSMC. This shift is characteristic of migratory, invasive, and contractile nature of VSMC. We measured extracellular matrix metalloproteinases (MMPs), tissue plasminogen activator (tPA), tissue inhibitor of metalloproteinase (TIMP), and collagen I expression in VSMC by specific substrate zymography and Northern blot analyses. The injured and elastin peptide, val-gly-val-ala-pro-gly, treated VSMC synthesized active MMPs and reduced expression of TIMP. The level of tPA and collagen type I was induced in the injured, invasive VSMC and in the val-gly-val-ala-pro-gly treated cells. To demonstrate the angiogenic role of elastin peptide to VSMC we performed in vitro organ culture with rings from normal coronary artery. After 3 days in culture the vascular rings in the collagen gel containing elastin peptide elaborated MMP activity and sprouted and grew. The results suggest that val-gly-val-ala-pro-gly peptide generated at the site of proteolysis during vascular injury may have angiogenic activity. J. Cell. Biochem. 75:515–527. 1999.

Key words: vascular smooth muscle cell; collateral; hypertrophy; fibrosis; invasion; migration; elastin peptide; angiogenesis; extracellular matrix metalloproteinase; tissue inhibitor; gene expression

Under normal physiological conditions, activation of smooth muscle cell function is regulated by the factors released by the endothelium; otherwise, smooth muscle cells are metabolically quite active [Bassenge and Busse, 1988]. Smooth muscle and endothelium are separated by a basement membrane and elastic laminae [Ross, 1993]. Extracellular matrix (ECM), particularly elastin in its three-dimensional relationship with integrin receptors surrounds the vascular smooth muscle cells [Hay, 1981]. Unless injury occurs, turnover of ECM components in the vessel wall are very low [Rucklidge et al., 1992]. Mechanical injury [Sipinga et al., 1997] and elevated circulating homocysteine [Rolland et al., 1995] initiate endothelial dysfunction and induce smooth muscle cell migration (contraction/relaxation) and proliferation. Mechanical injury to cultured cells has been used to study the effects of a stretch on tumor cells [Marshall et al., 1992], fibroblasts [Tsuboi et al., 1990], rabbit aortic muscle cells [James et al., 1993], and microvascular endothelial cells [Pepper et al., 1992]. Matrix degradation may initiate an angiogenic cascade during the development of existing collateral vessels and vasavasorum or initiating new vessels [Topol and Ellis, 1991; Symes and Sniderman, 1994]. A role for ECM synthesis and degradation, and proteinases has been demonstrated in cellular migration and wound healing [Folkman and Haudenschild, 1980; Tyagi et al., 1995a]. The role of ECM-degradation products (i.e., ECM dynamics) in vascular cell function is not well understood.

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Alterations in ECM composition induce cytoskeletal changes in the fibroblast cells [Carver et al., 1991]. Stimulation of collagenase secretion was observed in rheumatoid synovial tissue following addition of type-II and type-I collagen-derived peptides [Fisher et al., 1982]. Laminin (a member of ECM) and a synthetic peptide of 19 amino acids from the laminin A chain have stimulated the secretion of type IV collagenase (MMP-2) activity from human tumor cell lines [Kanemoto et al., 1990]. Expression of elastinolytic enzymes (including MMPs) in smooth muscle cells was increased by elastin-derived peptide [Ghuysen-Itard et al., 1992; Cohen et al., 1992]. Elastin peptides exert chemoattractive effects on human monocytes and fibroblasts [Senior et al., 1980]. Elastin-peptide regulates neutrophil elastase activity [Tyagi and Simon, 1993, 1994]. Proteinases exhibiting elastinolytic activity have been implicated in the disappearance of elastic fibers which occurs with aging [Szendroi et al., 1984]. Aortic smooth muscle cells secrete elastinolytic enzymes (MMP-2) in response to elastin-peptide [Ghuysen-Itard et al., 1992; Robert et al., 1984]. We demonstrated that collagen and elastin-peptides proliferate cardiac interstitial fibroblast cells and induce interstitial collagenase, MMP-1 [Tyagi et al., 1996a]. Elastin peptide, val-gly-val-ala-pro-gly, is repeated multiple times in the secondary structure of elastin molecule [Sandberg et al., 1981]. Also, this peptide is found in collagen [Fisher et al., 1982] as well as in laminin sequences [Kanemoto et al., 1990]. The elastin peptide, val-gly-val-ala-pro-gly, induces cellular proliferation and inhibits elastin synthesis in a feedback mechanism [Wachi et al., 1995]. Also, it has been demonstrated that this and related peptides adverse vascular tone [Faury et al., 1995; Kaibara et al., 1996; McEwan et al., 1986]. However, the role of val-gly-val-ala-pro-gly in vascular ECM remodeling is poorly understood.

Under physiological angiogenesis, vascular smooth muscle cells and in particular the endothelial cells experience different extracellular matrix environments which depend on whether they are in a resting state or they are undergoing sprouting and migration. Under normal conditions, quiescent smooth muscle cells rest in a specialized extracellular matrix environment which contains predominantly elastin, type I, III and IV collagens, and laminin. During angiogenesis and the formation of vasa vasorum they focally degrade their surrounding matrix components and subsequently migrate into the interstitial matrix of the surrounding connective tissue which consists mainly of type I collagen [Iruela-Arispe et al., 1991]. Matrix-degrading proteolytic enzymes which include neutral metalloproteinases, collagenase, and serine proteinases, plasminogen activators (PA) are involved in cellular migration [Moscatalli and Riffkin, 1988].

ECM is composed of proteinase/antiproteinase, elastin, collagen and proteoglycans and growth factors. During remodeling, proteinases breakdown ECM and release growth factors. Elastase released growth factors form ECM are implicated in collateral formation [Halperin et al., 1995]. Naturally, since elastin is a component of ECM, elastase and MMPs also release the elastin-peptide from ECM [Tyagi and Simon, 1993; Senior et al., 1991]. The role of growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in angiogenesis and collateral formation has been elaborated extensively [Montesano et al., 1986; Ladoux and Frelin, 1993]. ECM synthesis and degradation is the essential first step in the remodeling process which generates the elastin-peptide, val-gly-val-ala-pro-gly. It is known that the elastin-peptide is a stimulator of ECM synthesis [Szendroi et al., 1984; Tyagi et al., 1996a]. However, it is not known whether elastin-peptide is an angiogenic factor. In coronary collaterals [Angus et al., 1991; Tyagi et al., 1996b; Tyagi, 1997] and homocysteinemic aortas [Rolland et al., 1995], elastin peptide is elevated. It is possible that elastin-peptide stimulates VSMC proliferation, migration, and matrix invasion and matrix-degradation induces MMP expression. The results suggested that the elastin-peptide induces MMP, tPA, and collagen expression in VSMC, and plays a role in angiogenesis and vasculogenesis.

MATERIALS AND METHODS

Mechanical Injury to Human Coronary Artery

Human coronary arteries were obtained from an ischemic explanted heart. An Institutional Review Board waiver was obtained prior to isolation of coronary arteries from human heart. The vessels were used within 30 min of explantation. Injury to the vessels was performed by careful insertion of sterile razor blade through

the intima and media without disturbing the adventitia. The tissue section was incubated in serum-free minimum essential medium (MEM) for identification of in situ cellular migration/invasion. The cells from injured and non-injured vessel were isolated. The arterial tissue sections were incubated in 10% heat-inactivated fetal calf serum and mechanically stripped of endothelium and adventitia prior to isolation of the medial smooth muscle cells.

In Situ Tissue Morphometric Labeling

Tissue sections from human coronary arteries were prepared using standard histological techniques. Tissue was fixed with 10% buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H & E) for the tissue cellularity and Verhoeff's van Gieson for elastin [Bradbury and Gordon, 1982; Tyagi et al., 1995b]. Light transmission microscopy was performed at 2×, 20×, and 200× magnification.

Isolation of Human Vascular Smooth Muscle Cells (VSMC)

The VSMC from vascular media devoid of intima and adventitia were isolated by a modification of the combined collagenase and elastase digestion [Smith and Brock, 1998]. Medial smooth muscle cells were grown in 10% FCS [Tyagi et al., 1995c]. Isolated cells were free of endothelial cell contamination as determined by positive staining with human anti-smooth muscle actin-related antigen and negative staining for von Willebrand factor [Tyagi et al., 1995c]. The cells were characteristically "hill-and-valley" in morphology. The early passage (p-2) cells were used for most of the experiments. Cultures were routinely checked for the presence of mycoplasma [Chen, 1977] which has been shown to stimulate MMP level [Kluve et al., 1981].

VSMC were cultured on collagen-coated plates in medium which was supplemented with 20% fetal calf serum, 0.1% collagen suspension (Vitrogen 100, Celtrix, Santa Clara, CA), 2% normal rabbit serum, 4.5 mg/ml glucose, gentamycin, and fungizone (10 μg/ml) and 2 mM glutamine. For most experiments, cells were washed two times with serum-free Dulbecco's MEM and deprived of serum for 24 h prior to the experimental treatment.

Elastin-Peptide Induction of MMP, TIMP, and Collagen in VSMC

The VSMC were cultured in MEM with 10% FCS on Lab-Tek Permanox chamber slides (Nunc) or on 60-mm culture dishes. The cells were deprived of serum for 24 h. Cells were cultured with and without 100 μg/ml elastin-peptide, val-gly-val-ala-pro-gly (Sigma) in the serum-free medium for 24 h. The mRNA was isolated as described [Tyagi et al., 1995c].

Migration/Invasion Assays

Migratory and matrix invasive activity of VSMC was assessed by employing Boyden chamber apparatus as described [Boyden, 1962]. The PVDF filters (8-μm pore size, Nucleopore filters) were coated with a solution containing 100 μg/ml type I collagen (Vitrogen Corp) and 5 μg/ml fibronectin (CalBiochem Corp) and then air-dried. These filters were used in the Boyden chamber for migration of VSMC. For invasion assay synthetic basement membrane (BM) 10 μg was layered on these filters. The BM was prepared as described by Kleinman et al. [1986; Albini et al., 1987], using 12 mg/ml stock solution of BM matrix (Sigma). The coated filters and BM were allowed to dry at room temperature. The membrane was rehydrated with MEM prior to use.

In the upper chamber of Boyden apparatus 20,000 cells were suspended in MEM containing 0.1% BSA in 1 ml. The lower chamber contains chemoattractant (100 μg/ml elastin-peptide). The chamber were incubated for 4 h at 37°C in a 5% CO_2 atmosphere. The cells on both side of the filters were fixed and stained with hematoxylin/eosin. The cell on the underside of the filter were counted under ×400 magnification. Three fields were counted per filter, and all experiments were run in triplicate. The average of these determinations was taken as the mean for use in statistical comparisons. Each triplicate assay was repeated at least three times on a separate occasion with different VSMC preparations.

Experiments for migration and chemoattrative assays did not involve BM. Therefore BM was omitted from the filters. Using specific substrate zymography the medium from both chambers was analyzed for MMP activity after the experiment. The protein concentrations were determined by Bio-Rad dye-binding assay as described by Bradford [1976].

Electrophoresis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed with or without reduction by the method of Laemmli [1970]. After electrophoresis proteins were stained with silver nitrate or Coomassie Brilliant Blue R 250.

Gelatinolytic Activity

Matrix metalloproteinase activity in the gel was measured as described [Tyagi et al., 1993]. Denatured type I collagen, gelatin, fibronectin, or casein was added to standard Laemmli [1970] acrylamide polymerization mixtures at a final concentration of 0.5 mg/ml under non-reducing conditions. The gel was photographed and dried for permanent records. The activity was measured under identical amount of total protein loaded in the gel.

Stromelysin Activity

Stromelysin (MMP-3) is a good proteoglycanase. The MMP-3 activity was identified using 100 µg/ml fibronectin in the gel as described [Tyagi et al., 1993]. Identical incubation and staining conditions were employed for MMP-3 measurement as for gelatinase A and B. The samples were incubated with MMP-3 antibody (Binding Site Corp) prior to loading onto the substrate gels. The standard MMP-3 was isolated as described [Tyagi et al., 1995d] using fibronectin affinity chromatography and specific MMP-3 assays were carried out using fibronectin as the substrate in the gel.

Inhibition of MMP Activity

The N-terminal cysteine switch propeptide of MMP was synthesized and then used as the inhibitor of MMP activity. The sequence of the peptide natural (TMRKPRCGNPDVAN) and mutant (TMRKPRSGNPDVAN) were employed as active and inactive peptides, respectively. To avoid oxidation the cysteine peptide was stored as a dry solid under argon and dissolved with water immediately before the use.

Tissue Plasminogen Activator Activity

Proteins were electrophoretically fractionated on non-reducing SDS-PAGE (10%) gels and then cast with 0.4 mg/ml gelatin and 20 µg/ml human plasminogen. The gels were washed twice for 30 min in 2.5% Triton X-100 and then incubated for 16 h at 37°C in 50 mM Tris-Cl (pH 7.4) which contains $CaCl_2$. The gelatinolytic activity was detected by staining the gel for 2 h in 10% acetic acid, 15% isopropanol, 0.1% amido black, and then destaining the gel in a 10% acetic acid-20% methanol solution. The samples were incubated with anti-tPA antibody prior to loading onto the gel to identify specific tPA like activity along with standard tPA (CalBiochem Corp).

Northern Blot (mRNA) Analysis of MMP, TIMP, Collagen, and 18SR Gene

Total RNA was isolated from 1×10^6 cells using 4M Guanidine thiocyanate buffer [Churgwin et al., 1979]. RNA was quantitated at 260 nm absorbance. The purity of total RNA was assessed by absorbance ratio (260/280 nm) of 1.9. Twenty micrograms of total RNA were denatured in a formamide/formaldehyde solution at 65°C for 15 min and samples were then resolved on denaturing 1% agarose gel. The gel was transferred to nitrocellulose filter where it was prehybridized in a buffer containing 50% formamide, 5 ×SSC, 0.1% SDS, 5 ×Denhardts, 50 mM $NaHPO_4$, and 100 µg/ml denatured sperm DNA at 42°C for 4 h. Blots were then hybridized for 16 h at 42°C with [α-^{32}P]-dCTP random prime labelled cDNA. The membrane was washed in 0.1 M standard saline citrate plus 0.1% SDS at 42°C for 1 h and was exposed to X-ray films at −70°C for 24 h. The plasmid containing fibroblast collagenase (MMP-1) cDNA was obtained from American Type Culture Collection (ATCC). MMP-1 probe was a 2.05 kb Hind III and Sma I fragment from the human MMP-1 cDNA. MMP-2 probe was 2.119 kb EcoRI fragment from human MMP-2 cDNA. The plasmid containing TIMP-1 cDNA probe was obtained from Synergen Corp, CO. TIMP-1 probe was 0.7 kb EcoRI fragment of a human TIMP-1 cDNA. The collagen probe was 3.55 kb EcoRI fragment from a type I collagen α1-chain cDNA (ATCC). A 4.5 kb EcoRI fragment of 18SR gene (a gift from Dr. R. Guntaka) was used as an internal control. Bands on the autoradiographs were scanned. The relative intensity level of transcripts of MMP-1, MMP-2, collagen 1, and TIMP-1 were normalized with 18SR—an internal control.

Angiogenic Assay

Vascular culture was carried out using O-ring sections of normal coronary vessel. The vascular tissue was laid on the top of a thin

collagen gel matrix. The neutral pH collagen gel (2.8 mg/ml) was prepared from 12 mg/ml of acid soluble rat tail collagen (Sigma). The gels were prepared with and without the addition of elastin-peptide. The gel containing vascular tissue was incubated for 3 days in 5% serum. To inhibit MMP activity identical arterial tissue sections were incubated in the presence of active propeptide. The gel was stained and the MMP activity was visualized.

Statistical Analysis

The histographic data are expressed as mean ± SD. Difference between experimental condition of cell migration and invasion vs. normal cell activity were assessed by paired t-test. A value of $P < 0.05$ was considered significant.

RESULTS

Migration of Human Vascular Smooth Muscle Cells (VSMC) Following Injury

To demonstrate whether the injury to vascular elastin laminae initiates migration of medial smooth muscle cells, an insertion was made into the lumen of human coronary vessel through inner elastic laminal layers. The vessel was stained for elastin by van Gieson. Figure 1A,B elicit the breakdown of elastic membrane. The injured and uninjured tissues were cultured in 5% serum for 3 h at 37°C. The vessels were stained with H & E for the cellularity in the vessel wall. Following injury to the inner elastic laminae the smooth muscle cells are polarized and migrated towards the apical side in the vessel (Fig. 1C). There was no migration of the cells in the uninjured vessel (Fig. 1D). The shape of cells in injured vessels was apparently elongated. The cells were quiescent and roundish in the normal vessel. These results suggested activation of VSMCs following elastin breakdown in the vessel wall.

To identify the phenotypic-shift in the smooth muscle cells following elastin-breakdown, the VSMC from normal (Fig. 2A) and from injured coronary vessels (Fig. 2C) were isolated. The cells isolated from injured vessel demonstrated pseudopodial and invasive characteristics (Fig.

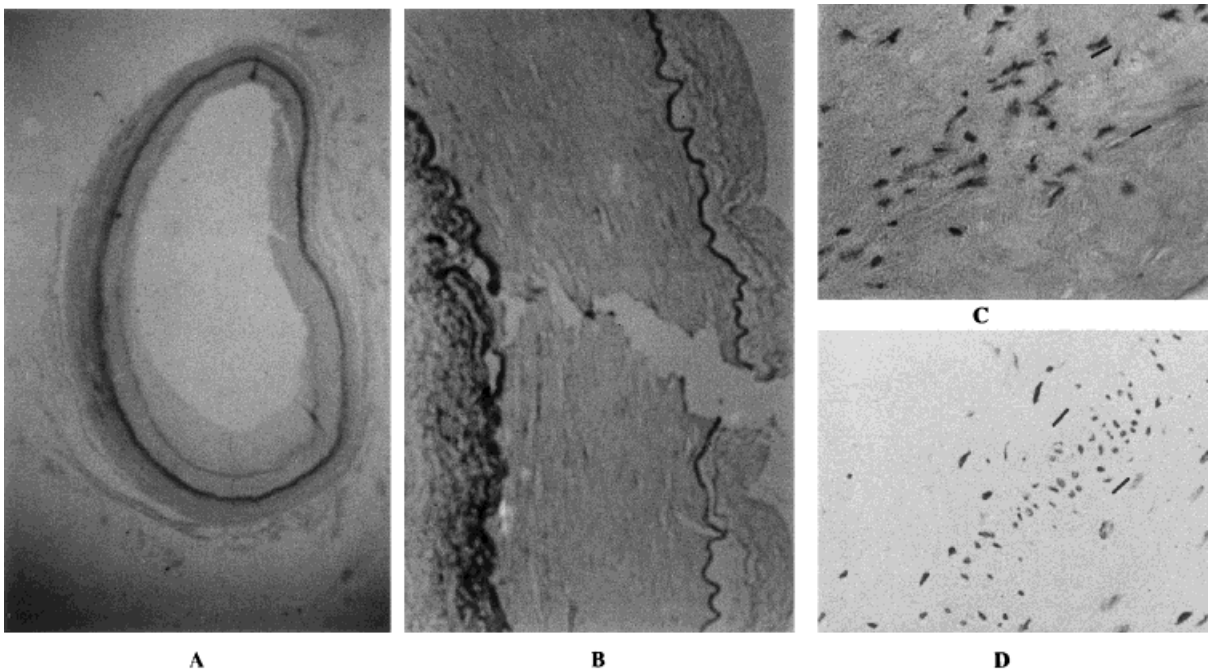

Fig. 1. Migration of human vascular smooth muscle cells following intimal-medial injury to coronary vessels: Coronary vessels were isolated from an ischemic cardiomyopathic human heart in which some coronaries were apparently normal intima was minimum. **A:** The intima was mechanically injured and stained with Verhoeff's van Gieson for elastin (2× magnification). The vessel was injured by cutting with a razor blade through the intima, inner elastic laminae, and media. **B:** The 200× magnification of (**A**), showing breakage of intima, inner elastic laminae, and media. **C:** The injured artery was organ cultured in 5% serum for 3 h following injury and stained with hematoxylin-eosin (200× magnification) to determine the cellularity of the tissue. An identical tissue section from uninjured tissue stained with H & E is shown in (**D**). Dash-bar distance show medial thickness. Note, migration and polarization of cells in injured tissue (**C**) as compared to uninjured vessel (**D**).

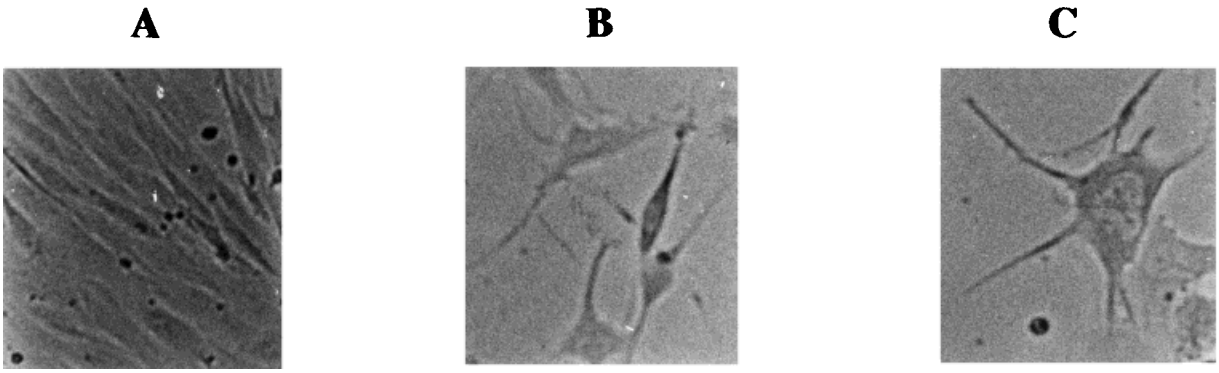

Fig. 2. Phase contrast light micrographs of VSMC (p-2) isolated from normal vessel (**A**); 3 h post injury to the vessel (**C**); and elastin-peptide treated (**B**) VSMC cells. A: VSMC isolated from the normal vessel, 20× magnification. **B**: VSMC were treated with elastin-peptide for 24 h, 200× magnification. **C**: Invasive VSMC isolated from injured vessel 3 h following injury (400× magnification). There were apparent phenotypic changes in the invasive and elastin-peptide treated VSMC.

2C). The VSMC treated with 100 µg/ml elastin peptide (Fig. 2B) are phenotypically similar to the VSMC isolated from elastin breakdown vessels. These results suggested that elastin breakdown product introduced contractile phenotypic shift in VSMC.

Migration and Invasion of VSMC in Response to the Chemoattractive Activity of the Elastin Peptide Through the Matrix Barrier

Cultured VSMC were placed in the upper chamber of a Boyden apparatus. The lower chamber contains the elastin peptide. These two chambers were separated by a filter and a matrix barrier of 10 µg reconstituted BM. The migrating cells undergone extensive cellular modifications in transversing the filter. The cells treated with elastin-peptide under go a similar modification (Fig. 2B). The VSMC migration through the BM is the response of chemoattractive activity of the elastin peptide in the lower chamber. The control with albumin instead of elastin peptide shows no migratory activity. Abolishing the chemoattractive gradient by placing elastin peptide in both chambers eliminated the cellular migration through the BM (Fig. 3). These results suggested that elastin peptide exerted a chemoattrative response to VSMC and induced migratory and invasive phenotypic changes.

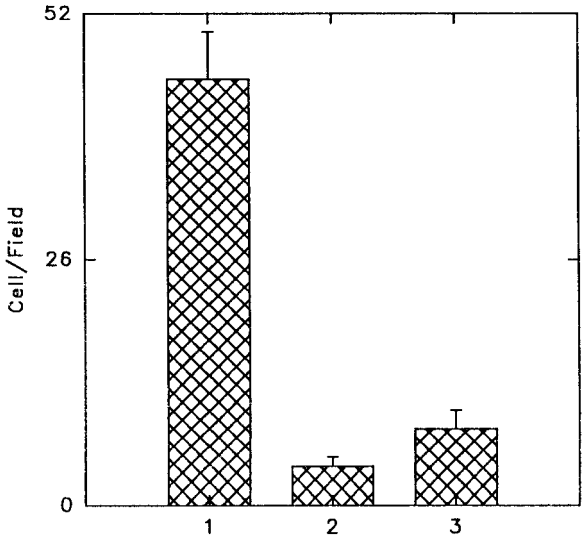

Fig. 3. Bar graph representation of chemoattractant activity of elastin-peptide to human vascular smooth muscle cells in the Boyden chamber apparatus. Boyden chambers were assembled by adding 10 µg/ml elastin-peptide or 0.1% bovine serum albumin (BSA) to the lower chamber. Filters were then coated with fibronectin/collagen I and supercoated with reconstituted basement membrane. The coated filters placed in the apparatus. Twenty-thousand cells were suspended in 1 ml DMEM containing 0.1% BSA or 10 µg/ml elastin-peptide and then were added to the upper chamber. After incubation at 37°C for 5 h, cells were fixed on the filter, stained, and counted at ×400 magnification. (**1**) indicates invasion with elastin-peptide in the bottom chamber only; (**2**) invasion with no chemoattractant (i.e., BSA in upper and bottom chambers); and (**3**) no invasion with elastin-peptide in both chambers in the Boyden apparatus and on both sides of the basement membrane matrix.

Gelatinase (MMP-2) Activity

To identify whether the migratory and invasive activity of VSMC is regulated by gelatinase activity, we measured MMP-2 activity by gelatin zymography (Fig. 4). The gelatinolytic activity was measured in the condition medium of invasive cells which were isolated from the injured vessel and the normal VSMC which were treated with elastin peptide. There is a basal activity of MMP-2 at 72–66 kDa (gelatin-

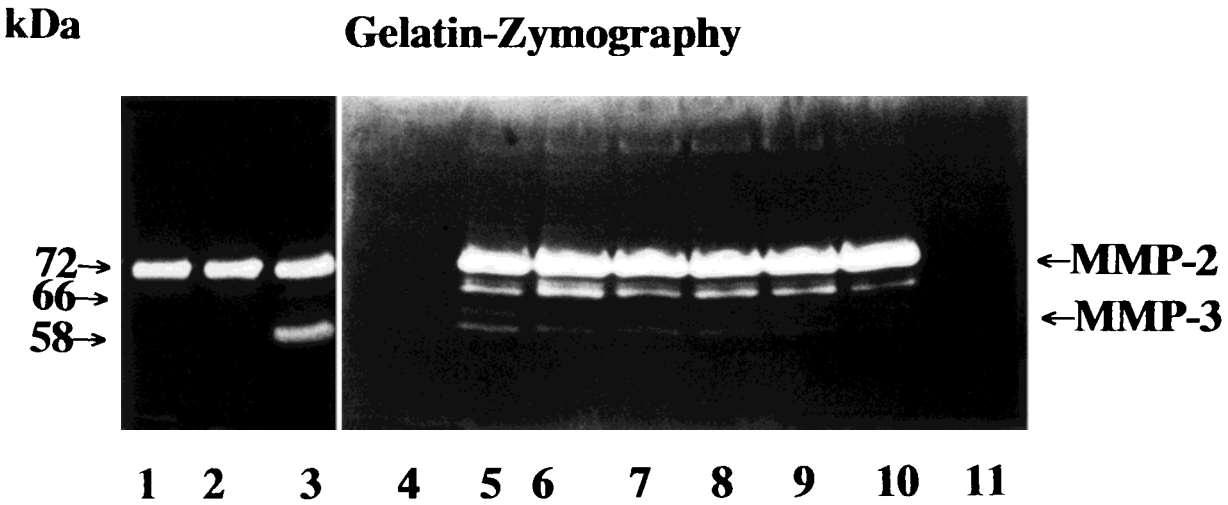

Fig. 4. Gelatin zymographic analysis of invasive and elastin-peptide treated VSMC: Cells (p-2) were cultured in serum-free medium for 24 h prior to treatment with elastin peptide. **Lane 1**, medium from normal control cells; **lane 2**, medium from control cells treated with elastin-peptide prior to loading onto the gel; **lane 3**, VSMC were treated with elastin-peptide for 24 hrs and the medium was then loaded onto the gel; **Lane 4**, medium was treated with phenanothroline prior to loading onto the gel; **lanes 5,6**, serum-free medium from invasive cells isolated from injured vessels; **lanes 7,8**, serum-free medium from invasive cells treated with inactive propeptide prior to loading onto the gel; **lanes 9,10**, serum-free medium of invasive cells treated with PMSF prior to loading onto the gel; **lane 11**, serum-free medium of invasive cells treated with active propeptide.

ase A) in normal VSMC. The elastin peptide has no effect on the MMP activity extracellularly (Fig. 4, lane 2). The treatment of elastin peptide induced gelatinase A activity and induced an extra band at ~58 kDa. The cells isolated from injured vessel demonstrated enhanced MMP-2 activity as well as an extra band at ~58 kDa. This activity was not inhibited by phenyl methyl sulfonyl fluoride (PMSF) or the inactive cysteine-switch propeptide the activity was completely blocked by phenanthroline and active propeptide. These results suggest the enhanced expression of gelatinase activity in the invasive and elastin peptide treated VSMC.

Stromelysin (MMP-3) Activity

To determine whether injury to the vessel and treatment of VSMC with elastin peptide induces stromelysin, we used a fibronectin substrate zymography (Fig. 5). MMP-3 is a proteoglycanase. In Figure 5 we demonstrated that fibronectin, a proteoglycan, is degraded and a band ~58 kDa is observed in the gel. Control (normal) VSMC show no stromelysin activity. The invasive VSMC isolated from injured arteries and VSMC treated with elastin peptide demonstrated MMP-3 activity. The proteoglycanase activity was abolished by pretreatment with anti MMP-3 antibody. Which suggests specificity of MMP-3 band in fibronectin gels (Fig. 5).

Tissue Plasminogen Activity

Most MMPs are secreted as the latent form and activated extracellularly. Plasmin can activate latent MMP at the neutral physiological conditions. To measure the tPA activity in the normal and invasive VSMC we performed plasminogen/casein zymography. The band at 66 kDa demonstrated tPA activity and not uPA (55 kDa). Furthermore, anti-tPA antibody abolished most of the tPA activity suggesting the presence of tPA in the invasive and elastin peptide treated VSMC (Fig. 6). This may suggest the role of tPA and activation of MMP in VSMC following injury.

Role of tPA and MMP in Invasion and Migration of VSMC

We tested the hypothesis that the MMP-2 and tPA are important in the invasiveness of VSMC during migration following tissue injury. Using antibody to neutralize tPA and active propeptide to inhibit MMP-2 in Boyden chamber assays, we demonstrated the effective role of MMP-2 and tPA in the invasive activity of these cells. Invasion was inhibited by $> 60\%$ ($P < 0.001$) by using anti-tPA antibody or cysteine-switch propeptide. There was no significant change in the migration of VSMC with or without the anti-tPA or propeptide. These results suggest specific role of MMP and tPA in the

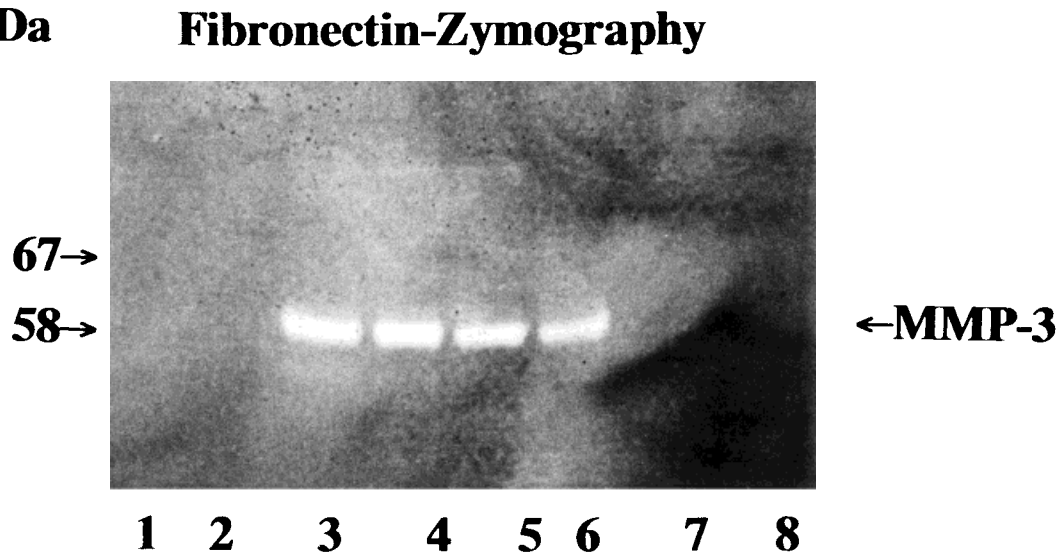

Fig. 5. Fibronectin zymographic analysis of invasive and elastin-peptide treated VSMC: Cells (p-2) were cultured in serum-free medium for 24 h prior to treatment with elastin peptide. **Lane 1**, medium from normal control cells; **lane 2**, medium from control cells treated with elastin-peptide prior to loading onto the gel; **lane 3**, VSMC were treated with elastin-peptide for 24 h and the medium was then loaded onto to the gel; **lane 4**, serum-free medium from invasive cells; **lane 5**, serum-free medium from invasive cell treated with anti-MMP-1 antibody prior to loading onto the gel; **lane 6**, standard MMP-3; **lane 7**, serum-free medium from invasive cells treated with anti-MMP-3 antibody prior to loading onto to the gel; **lane 8**, medium from elastin peptide treated VSMC treated with anti-MMP-3 antibody.

invasion of cell matrix barrier invasion and suggest no effect on cell migration (Fig. 7).

Steady-State mRNA Analysis of MMP-1, MMP-2, TIMP-1, and Collagen I

In order to demonstrate whether injury and elastin peptide induces the MMP and collagen expression at the transcription level, Northern blot analysis was performed using cDNA probes for MMP-1, MMP-2, TIMP-1, and type I collagen with RNA isolated from the invasive and the elastin peptide treated VSMC. The level of steady-state messenger RNA for MMP-1 and MMP-2 was increased three-fold ($P < 0.005$) in the invasive VSMC (Fig. 8). There was a small increase in the MMP-1 and MMP-2 mRNAs cells treated with elastin peptide. The level of TIMP-1 was significantly ($P < 0.005$) decreased in invasive and elastin peptide treated cells. The level of collagen I was significantly increased in the invasive as well as in the cells treated with elastin peptide (Fig. 8). These results suggested that during injury, migration and invasion VSMC synthesize collagen as well as MMPs and turn off TIMP-1 expression.

Angiogenic Activity of Elastin Peptide

To identify whether the induction in collagen I and collagenase expressions and reduction in TIMP-1 level is associated with development of new vessels or sprouting of existing vessels, we carried out an in vitro organ culture on the collagen gel matrix which was incorporated with and without elastin peptides. The lytic activity in the gel indicated MMP activity in the vessel which was cultured in the presence of elastin peptide (Fig. 9). This activity was inhibited by active cysteine-switch propeptide suggesting a role of MMPs in vessel sprouting which happens during angiogenesis initiated by elastin-degradation product.

DISCUSSION

Identification of the factors which participate in ECM degradation as well as their specific roles and interactions with smooth muscle cells may contribute to our understanding of vascular smooth muscle function during physiological angiogenesis. Angiogenesis requires cell migration and matrix barrier disruption. The physiological angiogenesis is dictated by the composition and organization of the extracellular environment. The understanding of interactions between the cell and ECM components will allow the relationship between structure (i.e., ECM) and the physiological function (i.e., angiogenesis, cell migration, contraction and invasion) to be elucidated. We demonstrated in

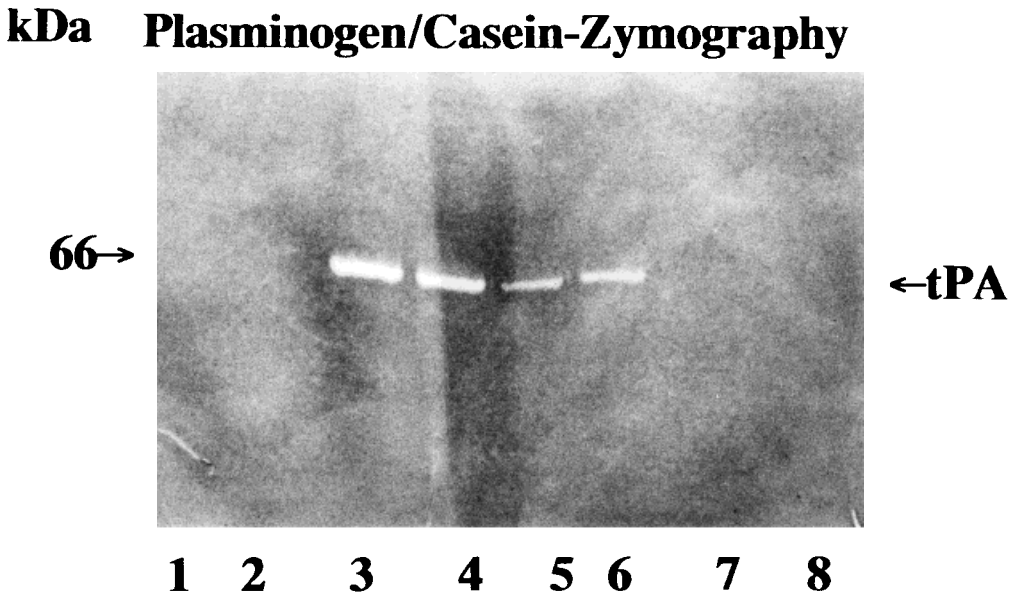

Fig. 6. Plasminogen/casein zymographic analysis of invasive and elastin-peptide treated cells: Cells (p-2) were cultured in serum-free medium for 24 h prior to treatment with elastin peptide. **Lane 1**, medium from control cells; **lane 2**, medium from control cells treated with elastin-peptide prior to loading onto the gel; **lane 3**, VSMC were treated with elastin-peptide for 24 h and the medium was then loaded onto to the gel; **lane 4**, serum-free medium from invasive cells; **lane 5**, serum-free medium from invasive cells treated with anti-tPA antibody prior to loading onto the gel; **lane 6**, standard tPA; **lane 7**, medium from invasive cells treated with anti-tPA antibody prior to loading onto to the gel; **lane 8**, medium from elastin peptide treated VSMC treated with anti-tPA antibody.

this report that invasive VSMC isolated from injured vessels and normal VSMC in response to elastin peptide invade a reconstituted BM as they migrate toward the chemical gradient of elastin peptide. These cells also undergo phenotypic changes following injury and the treatment of elastin peptide. Invasive and elastin peptide treated cells secreted active MMPs and tPA. These proteinases are involved in VSMC BM invasion. The invasive and elastin peptide treated VSMC also synthesize type I collagen and decreased level of TIMP-1. These results suggested a role of collagen and a decreased level of TIMP following migration and remodeling of VSMC in response to injury or ECM-degradation. The vessel in collagen gel matrix which contains elastin peptide in organ culture condition grow and sprout and produce active MMPs. These findings suggested a role of elastin-degradation in vessel growth and development.

MMP and TIMP are shown to be induced by injury, migration and proliferation of the cells [James et al., 1993; Pauly et al., 1994]. Level of collagenase and stromelysin was increased in vitro model of rabbit muscle cell injury [James et al., 1993]. Our results suggest that injury to the human vessel induced synthetic changes in the VSMC and produce active collagenase and stromelysin. We demonstrated that elastin peptide induces MMP-1, -2, and -3 in VSMCs. This suggested that the basement membrane degradation products, post-injury, induce MMPs and alter VSMC phenotype.

TIMP regulate MMPs post translationally [Goldberg et al., 1989]. Level of TIMP in migratory and invasive cells is found to be reduced [Stetler-Stevenson, 1990]. We observed significant reduction in the level of TIMP in injured or elastin treated VSMC. TIMPs have homology to some cytokines and have shown to inhibit cell proliferation [Moses et al., 1990]. This may suggest that reduced level of TIMP may unoppose VSMC proliferation and migration. To identify the mechanism responsible for invasive and migratory activity of VSMC and the role of MMPs and tPA, we tested the inhibitor of MMP and anti-tPA antibody. We observed that the migration was not inhibited in the presence or absence of MMP inhibitor or the anti-tPA antibody; however, the invasion of basement membrane by VSMC was inhibited by propeptide and anti-tPA antibody (Fig. 7).

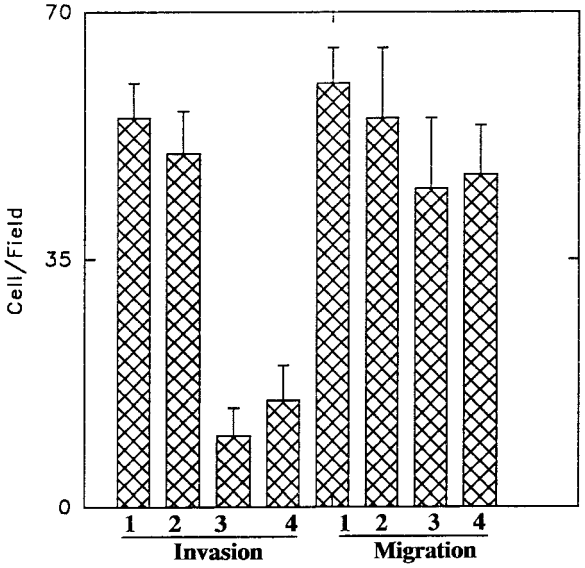

Fig. 7. Bar graph showing the effect of MMP propeptide and anti-tPA antibody on the migration and invasion of VSMC: The invasion through the 10 μm matrix layer and 8-mm-diameter pores of Nucleopore PVDF filter was measured. (**1**) control invasive cells; (**2**) invasion of basement membrane by the cells in the presence of inactive propeptide; (**3**) inhibition of invasion in the presence of active propeptide; (**4**) inhibition of invasion in the presence of anti-tPA antibody; The migration was measured in the absence of matrix barrier. (**1**) control migratory cells; (**2**) migration through the filter membrane by the cells in the presence of inactive propeptide; (**3**) migration in the presence of active propeptide; (**4**) migration in the presence of anti-tPA antibody ($P < 0.001$, n = 12).

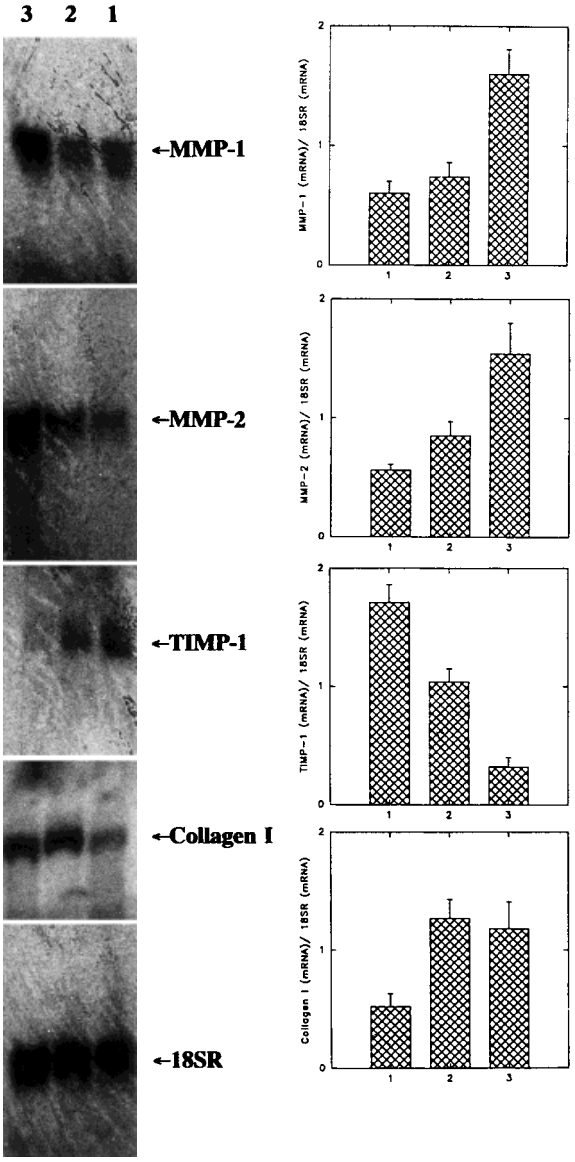

Fig. 8. Typical presentation of the expression of MMP-1, MMP-2, TIMP-1, collagen I, and 18SR mRNA in invasive and elastin peptide treated VSMC: VSMC alone (**1**); elastin-treated VSMC (**2**); and invasive cells isolated from injured vessel (**3**). Northern blots of total RNA (10 μg/lane) was probed for MMP-1, MMP-2, TIMP-1, and Collagen I. The 18SR gene was used as an internal control. The size of transcript is 2.4 kb for MMP-1, 3.5 kb for MMP-2, 1.5 kb for TIMP-1, 3.55 kb for collagen; and 4.5 kb for 18SR gene. The experiments were carried out in triplicates. Histographic presentation of corresponding scanned data normalized with 18SR gene is shown on the right side of the panel.

Transcription of type I collagen was initiated in aortic endothelial cells undergoing angiogenesis in vivo [Luisa Iruela-Arispe et al., 1991]. These authors suggested that extracellular matrix components are actively synthesized by endothelial cells undergoing angiogenesis [Luisa Iruela-Arispe et al., 1991]. Our results show that the level of collagen I mRNA is also increased in the injured VSMC and following elastin peptide treatment (Fig. 8). The co-increase in MMP expression with collagen may suggest a significant role of MMPs in collagen processing. Ingber and Folkman [1988] have described the metabolic reduction of collagen synthesis regresses capillary growth. Being a primary component of connective tissue collagen provides necessary substratum for VSMC and for the new vessel growth. Also, type I collagen in concert with cell surface receptor integrins might modulate a metabolic pathway in VSMC which will activate transcription of genes requisite to the process of angiogenesis.

Elastin-peptide inhibits Ca^{2+} loading into the vascular cells; concluding that the elastin-peptide inhibits the atherosclerotic process [Yosiyuki and Kuda, 1988]. Therefore induces the vasodilatation of coronary vessels. Also, elastin-peptide regulates Ca^{2+} mobilization in neutrophils [Varga et al., 1988] suggesting a functional role of the elastin-peptide in the vessel

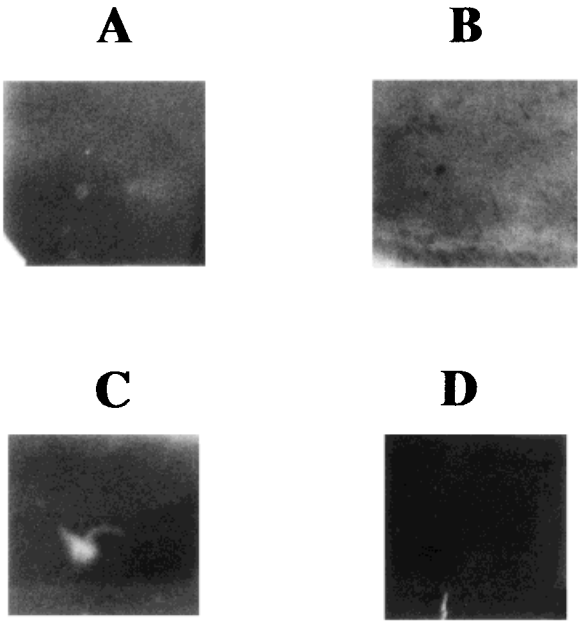

Fig. 9. Organ culture: angiogenic activity of elastin-peptide and MMP expression of the vessel: O-ring section of a freshly isolated microvessel from an ischemic heart was laid onto the collagen gel matrix containing elastin-peptide (**C**) or no peptide (**A**). The plate containing vessel and gel-matrix was incubated for 3 days in DMEM. The matrix gel was stained for MMP activity by coomassie blue. Identical corresponding sections were incubated in the presence of active propeptide (**B,D**).

walls. Elastin breakdown products have been observed in coronary collaterals and are unique to these vessels but the elastin breakdown products were not found in the non-collateral vessels [Angus et al., 1991]. The role of the elastin peptide in the functional properties of the collateral and non-collateral vessels is not known. These studies are in progress.

Perspective

The development of in vitro injury model and cell differentiation between migration and invasion will aid in determining the factors responsible for invasive and migratory function in VSMC since cell proliferation and migration are independent of MMP inhibitors [Bendeck et al., 1996]. Our assays will further the understanding of the differential role of proteinase, inhibitors, and ECM-degradation products in vascular remodeling following injury, arteriosclerosis, restenosis, collateralization, angiogenesis, and formation of vasavasorum.

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