Migration of Cultured Vascular Smooth Muscle Cells Through a Basement Membrane Barrier Requires Type IV Collagenase Activity and Is Inhibited by Cellular Differentiation

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Abstract The migration of vascular smooth muscle cells (VSMCs) from the tunica media to the neointima is a key event in the development and progression of many vascular diseases and a highly predictable consequence of mechanical injury to the blood vessel. In vivo, VSMCs are surrounded by and embedded in a variety of extracellular matrices (ECMs) that must be traversed during migration. One of the principal barriers to cell movement in the intact vessel is the basement membrane (BM) that surrounds each VSMC and separates the VSMC-containing medial cell layer from the endothelium. We have used a Boyden chamber to monitor the ability of VSMCs to degrade a BM barrier as they migrate toward a chemoattractant and to define the role of extracellular proteases in this process. We show that cultured VSMCs can migrate across a BM barrier and that this ability was dependent on the phenotypic state of the cell. VSMCs maintained in a proliferating or "synthetic" state readily migrated across a BM toward a chemoattractant, whereas the migration of serum-starved/differentiated VSMCs was suppressed by >80% (P<.001). By use of a number of peptides that inhibit matrix metalloproteinase (MMP) activity, the migration of proliferating VSMCs across the BM barrier was inhibited by >80% (P<.0001), whereas migration that occurred in the absence of the barrier was unaffected. Northern blotting and zymographic analyses indicated that 72-kD type IV collagenase (MMP2) was the principal MMP expressed and secreted by these cells. Accordingly, antisera capable of selectively neutralizing MMP2 activity also inhibited VSMC migration across the barrier without significantly affecting the migration of VSMCs in the absence of the barrier. Finally, MMP2 activity was also regulated by the phenotypic state of the cells in that MMP2 activity expressed by serum-starved/differentiated VSMCs was <5% of that measured in proliferating VSMCs. Extrapolating to the in vivo situation in which VSMCs reside in an ECM composed of various BM barriers, these results suggest that VSMC migration in vivo may be dependent on MMP2 activity. That activity, in turn, could be regulated by the phenotypic state of VSMCs and increase as these cells undergo the transition from a quiescent and differentiated state to that of a dedifferentiated, proliferating, and motile phenotype after injury to the vessel. (Circ Res. 1994;75:41-54.)

Key Words smooth muscle cells • chemotaxis • matrix metalloproteinases • extracellular matrix • basement membrane

Vascular smooth muscle cells (VSMCs) play a major role in the formation of arterial intimal lesions in atherosclerosis and are a major component of the accumulated fibrocellular mass in the neointima associated with restenosis following balloon angioplasty. Although some VSMCs reside in the tunica intima of normal human arteries that are susceptible to atherosclerosis, the majority of VSMCs reside in the tunica media, where they are quiescent and express a variety of differentiation-specific genes important for their function in the physiological regulation of vessel tone and blood pressure. A consistent finding in many if not all vascular disease states, however, is the extra-medial presence of VSMCs due to their migration from the tunica media to the tunica intima. This occurs when the vessel has been either mechanically injured (as during balloon catheter angioplasty) or infiltrated with monocytes and T lymphocytes (as during the inflammatory injury seen in the early stages of atherosclerosis). Unlike VSMCs that reside in the media, VSMCs that migrate to the intima initially exhibit a "synthetic" phenotype with a subcellular organization designed to support rapid cell growth and proliferation. Such cells may also secrete a fibrous extracellular matrix (ECM) and growth factors/cytokines that may influence the migration and differentiation of VSMCs remaining in the media. Although the factors responsible for initiating the phenotypic transformation of VSMCs and their migration have not been defined, growth factors or cytokines released by injured blood vessel cells or by circulating monocytes and macrophages are thought to play important roles in both events. A large body of in vivo and in vitro evidence, for example, strongly supports a role for platelet-derived growth factor (PDGF) as an important chemoattractant directing the migration of VSMCs toward the vessel intima.

VSMCs in the tunica media are surrounded by and embedded in a variety of different ECMs that must be...
breached during the migration of VSMCs from one tissue compartment to the next. Such barriers include the basement membrane (BM, consisting of collagen type IV, laminin, and heparan sulfate proteoglycans), which surrounds each VSMC in the intact vessel and separates the endothelial and medial cell layers. An interstitial matrix composed predominantly of collagen type I and fibronectin is the principal ECM material between VSMCs in the medial layer. In addition, multiple bands of elastic fibrils often separate concentric layers of VSMCs in the media. Although it has been reported that cultured VSMCs secrete proteases capable of degrading the ECM that they secrete, the type of matrix synthesized by cultured VSMCs can differ significantly from that which surrounds and encases them in vivo. Furthermore, the biochemical identity of the proteases involved in ECM degradation by VSMCs has not been established.

In many other cell types, the destruction of ECM barriers is mediated by extracellular proteases that are secreted by the migrating cell and that are generally localized to the immediate environment of the cell. Serylproteinases, cysteiny1proteinases, and matrix metalloproteinases (MMPs) are the principal extracellular proteases secreted by such cells. Both serine proteases and MMPs have been shown to be involved in tumor metastasis as well as in many physiological processes that involve ECM repair and remodeling. The principal extracellular serine protease involved in ECM remodeling is plasmin, which degrades a broad range of matrix molecules. Plasmin is secreted in a latent “pro” form (plasminogen) and is converted to its active form through the action of either the urokinase-type plasminogen activator (TPA) or tissue-type plasminogen activator (TAP). The actions of the plasminogen activators are opposed by endogenous plasminogen-activator inhibitors (PAIs). Medial VSMCs in vivo express and secrete PAI-1 and express low levels of both UPA and TPA. Both UPA and TPA activity increase after balloon injury to the vessel, with the changes in TPA expression closely correlating with the migration of medial VSMCs.

Like plasmin, the MMPs are also secreted in a pro form and are activated by cleavage of a short N-terminal segment that in the case of some MMPs is catalyzed by plasmin. The MMPs are a family of distinct proteases with differing specificities of cleavage toward various ECM components. These proteases contain a conserved catalytic domain and require metal ions, such as zinc, as cofactors. MMP1, for example, encodes an interstitial collagenase that disrupts the native structure of collagen I fibrils. These degraded fibrils can then be further digested by other MMPs called gelatinases. Both MMP2 and MMP9 possess gelatinase activity as well as specific degradative activity toward collagen type IV. Other MMPs are involved in the degradation of large glycoproteins, such as fibronectin and laminin, and the degradation of elastin fibrils. Human and primate VSMCs have been shown to constitutively express MMP2 in culture and to upregulate the expression of MMP1 gene in response to cytokines of the type shown to be released at the site of vascular injury. Endogenous inhibitors of MMP activity also exist and are secreted by the same cell types that secrete and activate MMP expression. Unlike plasmin, the expression of MMPs and their potential role in cell migration have not been previously addressed.

Isolated rat VSMCs cultured on a gel of reconstituted BM proteins initially organize themselves to form reticular networks. With time, these cells degrade the matrix and penetrate the underlying plastic, indicating that these VSMCs can secrete the proteases necessary to degrade complex ECMs, such as BMs. We have used an in vitro assay to monitor and manipulate the ability of VSMCs to degrade defined ECM barriers as they migrate toward a chemoattractant. For the chemoattractant, we chose PDGF-BB because of the abundance of evidence that this growth factor acts as a VSMC chemoattractant in vivo. We then monitored the migration of cells both in the presence (referred to hereafter as invasion) and absence (referred to as migration) of a BM barrier. We show here that migration across a BM (ie, invasion) was dramatically inhibited by inhibiting MMP activity with a peptide whose sequence was derived from the conserved “cysteinexwitch” region of the pro fragment of all MMPs and that was shown to inhibit MMP activity seen during gelatin zymography and in a fluorescent peptide assay for MMP activity. Migration in the absence of the BM barrier was unaffected by the peptide. Gelatin zymography and Northern blotting analyses indicated that synthetic VSMCs in cell culture synthesize and secrete an active form of MMP2 (72-kD type IV collagenase [gelatinase]), an MMP with an enzymatic specificity consistent with BM destruction. Neutralizing antisera to MMP2 inhibited VSMC migration through the BM barrier, while only slightly affecting migration in the absence of the barrier. The migration of “differentiated” VSMCs across both the BM membrane and in its absence was significantly suppressed, suggesting that cell differentiation altered the ability of the VSMCs to respond to PDGF-BB as a chemoattractant. In addition, MMP2 in the conditioned media of differentiated VSMCs was 15- to 20-fold less active than in synthetic cells. The acquisition of the ability to respond to a chemoattractant and the synthesis of enzymatically active proteases capable of degrading ECM barriers may, therefore, be part of the phenotypic transformation of VSMCs that occurs in response to vascular injury.

Materials and Methods

Cell Culture

Thoracic aortas from 3-month-old Wistar rats were removed and stripped of endothelium and adventitia. Medial VSMCs were obtained by modification of the combined collagenase and elastase digestion method. Cells were maintained in Dulbecco’s modified minimal essential media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mmol/L nonessential amino acids, 20 mmol/L L-glutamine, 50 mg/mL penicillin, 50 mg/mL streptomycin, and 10 mg/mL neomycin (GIBCO Laboratories) in a humidified 5% CO2 atmosphere at 37°C. Cells were passaged after reaching confluence using 0.25% trypsin/0.05% EDTA (National Institutes of Health Media Unit). Cells for the experiments described in this study were used between the 5th and 14th passages. Proliferating cells were harvested for migration assays or biochemical analyses at subconfluence. In vitro differentiation of VSMCs was accomplished by allowing cells to accumulate postconfluently to achieve the characteristic “hill-and-valley” morphology and then reducing FBS in the medium to 0.5% or replacing it with DMEM and GMS (1
μg/mL insulin, 0.67 ng/mL sodium selenite, 0.55 μg/mL transferrin, and 11 μg/mL sodium pyruvate) (GIBCO Laboratories) for 5 to 7 days.

Migration/Invasion Assays

Migration/invasion assays were performed with a modified Boyden chamber as previously described.27 For migration in the absence of a BM barrier, PVPF filters (8-μm pore diameter, Nucleopore filters) were first coated with a solution containing 100 μg/mL type I collagen (UBI) and 5 μg/mL fibronectin (Collaborative Research) and then air-dried. For migration in the presence of the BM barrier (defined here as invasion), reconstituted BM (10 μg) was then layered onto each coated filter and allowed to dry at room temperature. The membrane was then rehydrated with DMEM before use. This procedure produces a thin, continuous, and even coating of matrix material of ~10 μm in thickness. Reconstituted BM was prepared from the Engelbreth-Holm-Swarm tumor as described previously.28 Protein concentration of the extract was determined by the method of Bradford.29 The concentration of protein in the BM used in these experiments was ~8 mg/mL.

Boyden chambers were assembled by adding 10 ng/mL PDGF-BB in DMEM to the lower (chemoattractant) chamber. Cells (200,000) suspended in DMEM containing 0.1% bovine serum albumin (BSA) were then added to the upper chamber in a solution containing 100 μg/mL type I collagen (UBI) and 5 μg/mL fibronectin (Collaborative Research). The cells in the upper chamber were then incubated for 4 hours at 37°C in a 5% CO2 atmosphere. At the end of the incubation period, the cells on both sides of the filter were fixed and stained with hematoxylin/eosin (HEMA 3 differential staining system, Curtin Matheson Scientific). Usually, the cells on the upper surface were mechanically removed, and the cells remaining on the underside of the filter were counted under x400 magnification. In some cases, however, the cells on the upper surface were counted to determine whether changes in cell attachment accompanied phenotypic changes in the VSMCs or their treatment with various reagents. In all cases, four 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 separate occasions with different VSMC preparations.

Chromatix was also assayed in the Boyden chambers described above, except that the reconstituted BM was omitted. Supernatants from both chemooinvasion and chromatix upper chambers were removed after 4 hours for zymography and the collagenase peptide assay.

Inhibition of MMP Activity

A synthetic peptide capable of inhibiting MMP activity both in vitro and in vivo was used to determine whether MMPs play a role in the process of ECM invasion by VSMCs.30 The sequence of the peptide (TMRKPGRGNPDVAN) is based on the conserved cysteine-switch region present in the pro fragment of all MMPs and is thought to be responsible for inhibiting enzyme activity when the MMP is in the pro form.31 A peptide (TMRKPGRGNPDVAN) in which the cysteine at peptide position 7 is replaced by serine was used as a control for the study. These peptides are referred to throughout the text as MMP propeptides. Both peptides were synthesized using Boc butyloxycarbonyl methodology (Multiple Peptide Systems) and then deprotected in hydrogen fluoride in the presence of p-thiocresol to protect the sulfhydryl groups from oxidation. The composition and concentration of the peptides were confirmed by independent amino acid composition analyses. Because the "cysteine"-containing MMP propeptide is susceptible to oxidation, both peptides were stored as dry solids under argon and reconstituted with water immediately before their addition to the upper chamber of the Boyden apparatus. The effectiveness of the propeptide at inhibiting MMP activity was demonstrated by its ability to inhibit MMP development in the zymogram assay (Fig 3A) and MMP activity in the fluorescent peptide assay (Tables 1 and 2).

Two sets of commercially available thiol-based metal-bind- ing peptides that specifically inhibit collagenase were also tested for their ability to block invasion.32 One set is referred to as the phenylalanine derivatives: the “active” free thiol form being HS-CH2-R-CH(CH3)-CO-Phe-Ala-NH2 and the “inactive” protected form being Ac-S-CH2-R-CH2(CH3)2-CO-Phe-Ala-NH2. The other set is referred to as the naphthylalanine derivatives: the active free thiol form being HS-CH2-R-CH(CH3)2-CO-Nal-Ala-NH2 and the inactive protected form being Ac-S-CH2-R-CH2(CH3)2-CO-Nal-Ala-NH2. Both sets of inhibitors were obtained from Peptides International and were handled with the same precautions used for the MMP propeptides.

Two different rabbit antisera to human 72-kD type IV collagenase (MMP2) were used to neutralize or immunoblot MMP activity (antisera were generously provided by W. Stettler-Stevenson, National Cancer Institute, Bethesda, Md). Antibody Ab IVase was used for neutralization of MMP2 in the zymography (Fig 5A) and migration assays (Fig 5B). Nonimmune rabbit serum (Jackson Laboratories) at a protein concentration equivalent to that of the antisera was used as a negative control for the effects of Ab IVase. Both specific and nonimmune sera were partially purified before use by passage over protein A agarose and were added to the upper chamber of the Boyden apparatus along with the cells used for analysis.

The second antibody used in these studies was a peptide antisera called Ab 45. This antisera was affinity-purified and used for immunoprecipitation studies (Fig 8) and for depleting conditioned media of 72-kD gelatinase (Table 2). To control for possible nonspecific actions of the antisera, a 10-fold excess (wt/wt) of the peptide used in immunogen was preincubated with antisera before its use in the experiments.

Electron Microscopy

Scanning electron micrographs of the cells on the underside of the filter in the Boyden apparatus were prepared by first fixing the cells in a solution containing 4.0% formaldehyde and 2.5% glutaraldehyde for 4 hours and then transferring them to a solution containing 2% osmium tetroxide and 1% thio-carbohydrazide for 60 minutes. The latter solution was changed once during the incubation period. The cells were then dehy- drated through a graded series of ethanol. Filters were scanned with an Amray 1000 electron microscope by an independent contractor (Paragon Biotech).

Collagenase Zymography and Activity Assays

Gelatin-degrading proteinases secreted into the culture media by VSMCs were analyzed by nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels containing 0.1% (wt/vol) gelatin32 (Novex Chemical). Samples were denatured at room temperature in an equal volume of 0.25 M Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, and 10 μg/mL bromphenol blue. After electrophoresis, the gelatinases were renatured by incubating the gel at room temperature two times for 30 minutes in 2.5% (vol/vol) Triton X-100 and then at 37°C for 18 hours in 50 mmol/L Tris-HCl (pH 7.6), containing 0.2 mol/L NaCl, 5 mmol/L CaCl2, and 0.02% Brij 35 (wt/vol). The gels were stained for 90 minutes with 0.5% Coomassie brilliant blue G-250 and destained with 10% acetic acid in 40% methanol. Gelatinolytic activity was evident as a clear band against the blue background of stained gelatin. Assignment of bands to active and latent forms of MMP2 was made after in vitro activation by p-aminophenylmercuricacetate (APMA) and by reference to purified latent and APMA-activated recombinant human MMP2 purified from vaccinia virus–infected cells (gift of Rafi Fridman, Wayne State University).

Collagenase activity was measured using a modification of the X-methyl anthranilic acid (XMA) fluorescent peptide
assay.34 Briefly, 250 μL of conditioned media from VSMCs was concentrated to 10-fold with a Centricon 30 microconcentrator according to the manufacturer’s instructions (Amicon) and then incubated with 50 μg/mL of the peptide DNP-Pro-CHA-Gly-Cys(Me)-His-Ala-Lys(NMA)-NH2 (Peptides International), where CHA is cyclohexyl acid, in an assay buffer consisting of 200 mM/L NaCl, 50 mM/L HEPEs (pH 7.6), 5 mM/L CaCl2, 20 μM/L ZnSO4, and 0.05% Brij 35 in a total volume of 50 μL. After incubation for a defined time (typically 4 to 24 hours), the reaction was diluted to 1.5 mL with the assay buffer and read on a Perkin-Elmer spectrofluorometer with an excitation setting of 340 nm and an emission setting of 440 nm. The spectrofluorometer was routinely blanked on a sample containing only the buffer and peptide. Recombinant human MMP2 was used as a positive control in all assays and was activated by a 60-minute incubation at 37°C in 1 mM/L APMA. Activation of the enzyme was monitored by zymography as described above. The development of fluorescence was extract and time dependent and in the case of recombinant MMP2 was significantly enhanced by APMA pretreatment. Virtually all of the activity was inhibited by either 10 mM EGTA or 5 mM/L 1,10-phenanthroline and removed by selective immunoprecipitation of MMP2 with the affinity-purified Ab 45. The units of activity reported are relative to cell number and are expressed as relative fluorescent units.

**Immunoprecipitation of Metabolically Labeled MMP2**

The media covering VSMC cultures were removed, and the plates rinsed twice in DMEM alone and then incubated in DMEM containing 50 μCi/mL of L-[35S]methionine (translational grade, NEN) for 8 hours. The conditioned medium from such cultures was then mixed with an equal volume of 1% Triton X-100 and 0.1% BSA in phosphate-buffered saline. An aliquot (0.4 to 1 mL) of this was mixed with 10 μg Ab 45 (generously provided by W. Stetler-Stevenson, National Cancer Institute, Bethesda, Md.) and incubated overnight at 4°C. Protein A conjugated to agarose was then added to precipitate the immune complexes, which were then consecutively washed in 1% Nonidet P-40, 150 mM/L NaCl, 20 mM/L Tris-HCl (pH 8.0), 10% glycerol, 2 mM/L EDTA, 0.5% Nonidet P-40, 0.5 mol/L LiCl, 50 mM/L Tris-HCl (pH 7.5), 0.5 mol/L LiCl, and 50 mM/L Tris-HCl (pH 7.5)/10 mM/L Tris (pH 7.5). An equal volume of 2× SDS sample buffer (0.25 mol/L Tris-HCl [pH 6.8], 20% glycerol, 2% SDS, 10 mM/L 2-mercaptoethanol, and 10 μg/mL bromophenol blue) was then added and boiled for 3 minutes, and the samples were separated by SDS-PAGE on a 10% separating gel. After electrophoresis, the gel was fixed in 50% methanol/7% acetic acid and then prepared for gel fluorography according to the manufacturer’s instructions (ENHANCE; NEN). Immunoprecipitation was quantified with a Betascope 603 blot analyzer (Betagen Corp) and represented as counts per minute per 100,000 cells. Cell number was calculated from the total protein content of the cellular layer and the following relation: 10^10 cells = 0.76 mg protein, which was empirically derived in the laboratory by comparing cell number (determined by flow cytometry) and total cellular protein in duplicate cultures of proliferating and differentiated VSMCs. No significant difference was noted in the relation for proliferating and differentiated VSMCs.

**RNA Analyses**

Total RNA was isolated from VSMC cultures using the guanidinium isothiocyanate procedure.35 For Northern analyses, the RNA was fractionated on 1% agarose-formaldehyde gels after denaturing in mercaptoethanol. The fractionated RNA was electrophoretically transferred to a nylon membrane (Duralon, Stratagene Cloning Systems) in 1× Tris-acetate-EDTA buffer at 6 V/cm for 4 hours and then covalently cross-linked to the nylon membrane by UV irradiation (120 mJ). For most probes, the nylon blots were prehybridized for 2 to 18 hours in 50% formamide, 5× Denhardt’s solution,36 0.5% SDS, 0.1% NaPO4, 50 mM/L Tris-HCl (pH 8), 0.5 mM/L EDTA, and 200 μg/mL denatured salmon sperm DNA at 42°C and then hybridized in fresh solution of the above containing 1×10^5 cpm of probe per milliliter of hybridization solution. For α-smooth muscle actin mRNA, the blots were prehybridized and hybridized in Church buffer37 containing 100 μg/mL denatured salmon sperm DNA, 100 μg/mL yeast tRNA, 10 μg/mL polyadenylic acid at 60°C to 65°C. CDNA probes for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), total rat actin, rat 72-kD type IV collagenase, rat tissue inhibitor of metalloproteinase-1 (TIMP1), human tissue inhibitor of metalloproteinase-2 (TIMP2), and rat transferrin 1 and 2 were generated by polymerase chain reaction (PCR) using published nucleotide sequences and reverse-transcribed mRNA from either adult rat aorta (GAPDH and actin), rat 6R cells (72-kD type IV collagenase and rat TIMP1), HT 1080 cells (human TIMP2), or rat 6L cells treated with 12-O-tetradecanoylphorbol 13-acetate (PMA, 100 ng/mL for 24 hours) or epidermal growth factor (20 ng/mL for 8 hours) (rat transferrin 1 and 2, respectively). All of the primers used for cloning these probes were as follows: for rat GAPDH,38 sense AGCAACTCCCATCTCTCCA and antisense GATGGATTGCGAGAGAAC; for total actin,39 sense TGAATCAAGGAAGCCTT and antisense AACTGAAAGGCCTGATCCACA; for 72-kD gelatinase,40 sense TTTGATGACATGAG and antisense GGGAGCTCGAGCGAGAA; for TIMP1,41 sense ACCACCTTTACACCCGTTA and antisense AACACGAGGAAAAACTGTTGCA; for TIMP2,42 sense AGTTGAGGCTTAGAAACCCGCA and antisense GTACTGCGATTCTCTTGAAG; for transferrin 1,43 sense GAAAGATCATGAGGGCTC and antisense CAAACACTCACACAGAG; and for transferrin 2,44 sense CTGTATTATCTCATGATGCAAGACA and antisense GATGCACCTCTCCTACATTGT.

The PCR products were cloned into either the pCR1000 or pCRII vectors according to the manufacturer’s instructions (Invitrogen Corp) and then sequenced in full using the dyeodeoxy method to verify that the cloned products completely matched the published DNA sequence. cDNA probes for 92-kD collagenase44 and rat interstitial collagenase45 were generously provided by Dr. Gregory Goldberg (Washington University, St. Louis, Mo) and Dr. John Jeffrey (SUNY, Albany, NY), respectively. All cDNA probes were labeled by the random-primer method46 and then incubated with the membrane in fresh hybridization solution at 1×10^5 cpm/mL. The α-smooth muscle actin probe was a 35-base oligonucleotide complementary to sequences in the 3' untranslated region. Derived from the published data, its sequence was as follows: CCGCCTCTGTCTCAGACACAACTTGTTAATG.39 This oligonucleotide was labeled with α-[32P]dATP at its 3' end by use of terminal transferase.36 The mRNA levels for smooth muscle-specific myosin heavy chain (SMHC-1) and GAPDH were analyzed by quantitative PCR using the following oligonucleotide primers derived from published sequence data: for SMHC-1,47 sense GCCAGAGGAAAGGAACACCA and antisense TCAGCTGCTGAGGCTTTGT; and for GAPDH,48 sense AGCAACTCCCATCTCTCCA and antisense GATGGATTGCGAGAGAAC. PCR was run for 20 cycles at an annealing temperature of 60°C, and the products were separated by electrophoresis in a 1.5% agarose gel, denatured, transferred to nylon membranes (Genescreen Plus, Dupont) by capillary blotting in 10× standard saline citrate, and then hybridized with radiolabeled cDNA probes to human GAPDH (Clontech Laboratories) or rat SMHC-1. The latter probe was generated by PCR using the published nucleotide sequence47 and subcloned into the pCR1I vector (Invitrogen Corp), and then its nucleotide sequence was determined to ensure that it corresponded to the cDNA fragment to SMHC-1. The GAPDH primers yield an expected PCR product of 325 bp, whereas the SMHC primers
can yield two PCR fragments, a 442-bp fragment corresponding to the SM-1 MHC isofrom and a 481-bp fragment corresponding to the SM-2 SMHC isofrom. The hybridization of radiolabeled probes was quantified on a Betascope 603 Blot Analyzer (Betagen Corp.).

Statistical Evaluation

All values in the figures, table, and text are expressed as mean±SEM. Student’s unpaired t test was used to compare means between groups. A value of P<.001 is cited in the text as being statistically significant.

Results

VSMCs Invade Reconstituted BMs

Cultured VSMCs from the rat thoracic aorta or carotid artery were placed in the upper chamber of a Boyden apparatus, which was separated from the lower chamber containing the chemoattractant PDGF-BB by a filter and a barrier of 10 μg reconstituted BM. A cross section through a Boyden chamber filter-coated with reconstituted BM is shown in Fig 1, top. The coating (BM) created a uniform barrier that in the field shown was degraded directly over a pore (P1) through which a VSMC had apparently migrated. In contrast, the coating over other pores (eg, P2) not adjacent to any migrating cells remained intact. A scanning electron micrograph of the underside of the filter (Fig 1, bottom) showed attached VSMCs that were much larger than the diameter of the pores, indicating that they had undergone extensive cellular modifications in traversing the filter. These micrographs show that VSMCs can effectively migrate toward PDGF through a BM barrier and suggest that this migration is accompanied by local degradation of the matrix in the pathway of migration.

The number of VSMCs migrating across the BM barrier was dependent on the presence of a gradient of chemoattractant (Fig 2). Its removal from the lower chamber and replacement by a nonspecific agent, such as BSA, resulted in a dramatic reduction in the number of cells appearing on the underside of the filter (filled versus open bars in Fig 2), as did adding equal amounts of PDGF to both sides of the barrier (no gradient, cross-hatched bar in Fig 2). The number of cells appearing on the underside of the chamber increased with time, although fewer cells appeared in the first hour compared with later times. This reflected the fact that cell attachment was not immediate and required at least 1 hour for completion. To determine whether differential adhesiveness of cells affected any of the results described below, the time course of cell attachment was checked for each of the manipulations described below and found not to vary significantly among the different conditions tested or treatments used.

MMPs Are Required for VSMC Chemoinvasiveness

To determine if MMPs are required for VSMC migration through the reconstituted BM, we used two types of synthetic peptides that inhibit MMP activity by two different mechanisms. Inhibition by the first of these (the MMP propeptides) is based on highly conserved amino acid sequences in the pro segment of “latent” MMPs that are thought to mediate inhibition of enzymatic activity of the proenzyme form.31 By mimicking this pro segment, the peptide inhibits the activity of activated MMPs in which the pro segment has been cleaved. Such an MMP propeptide has been shown to effectively inhibit MMP activity in vitro and to inhibit tumor cell metastasis in vivo.30 Its efficacy in our system was confirmed by its ability to inhibit the development of MMP activity on a zymogram of conditioned media from VSMCs (Fig 3A) and to inhibit MMP activity in a fluorescent peptide assay (Tables 1 and 2). When added to VSMCs in the upper chamber of the Boyden apparatus, the peptide inhibited VSMC invasion (defined as migration through the BM barrier) by >80% (active MMP peptide in Fig 3B) while not significantly affecting migration in the absence of the BM barrier. A dose-response curve showed that 50% maximal inhibition of chemoatraction occurred at 20 μmol/L peptide, a concentration similar to that reported for the inhibition of in vitro tumor cell invasion.30

The specificity of this inhibition was demonstrated by incubating the VSMCs with a peptide that differed at only one amino acid, the cysteine in position 7 being changed to serine. As predicted from the cysteine-switch hypothesis34 and shown by others,35 replacement of the cysteine creates a minimally altered peptide with little ability to inhibit MMP activity (Fig 3A and Tables 1 and 2). Consequently, incubation of VSMCs with the cysteine-substituted peptide had no significant effect on migration in either the presence or absence of the BM barrier (inactive MMP peptide in Fig 3B).

The second set of peptide inhibitors were the thiol-based peptide inhibitors described by Darlak et al.32 These peptides coordinate zinc, which is required for MMP enzymatic activity. Their selectivity resides in the fact that they mimic the sequence of the active site. Two sets of these inhibitors were tried: one in which phenylalanine was the penultimate amino acid and another in which naphthylalanine resided in this position. The data in Table 1 compare the efficacy of these peptides and the MMP propeptides at inhibiting the activity of recombinant human MMP2 in a test tube and the migration of VSMCs in the presence (invasion) and absence (migration) of the BM barrier. Both thiol-based peptide inhibitors inhibited in vitro MMP activity as well as invasion but had no effect on migration in the absence of the BM barrier. Control peptides in which the thiol group was protected and no longer capable of metal binding had no effect in vitro MMP activity or VSMC invasion.

Since all these peptides can inhibit all MMPs, we used zymographic and Northern blotting analyses to determine which MMPs were expressed by the VSMCs under the conditions of our experiments and were the potential targets for inhibition by the peptides. Gelatin zymograms of VSMC-conditioned media from the upper well of the Boyden chamber showed that the major MMP expressed by these cells was MMP2 (Fig 4A), which was present mostly in the latent form. Lesser amounts of 92-kD type IV collagenase (MMP9) were also observed in the zymogram, although most if not all of this originated from enzyme present in the reconstituted BM used for coating the filters.46 Small amounts of cleaved (active) enzyme (migrating at 68 kD and below) could be seen only in proliferating (synthetic) VSMCs. The level of expression and activation of these proteases as determined by gelatin zymography was unaffected by PDGF (Table 2).
Since gelatin zymography is limited in its ability to detect many MMPs, we used Northern blotting analysis of total RNA isolated from VSMCs cultured under conditions similar to those used in the invasion assays to confirm the zymogram findings and to search for expression of other MMPs. These results confirmed that the major MMP expressed by these cells was MMP2 (Fig 4B). The mRNAs encoding MMP9 (Fig 4C), the interstitial collagenase (MMP1) (Fig 4D), or stromelysins 1 and 2 (MMP3 and MMP10) (Fig 4, panels E and F, respectively) were not detected in total RNA from the VSMCs. Appropriate controls (lanes 1 through 4) were included on each Northern blot to show that if the mRNAs for these proteases were present, they would have been detected.

On the basis of these findings, we directly tested the importance of MMP2 in VSMC invasiveness by using an antiserum to neutralize its activity in the Boyden chamber assays. The effectiveness of this reagent in neutralizing MMP2 activity was confirmed by its ability to
inhibit zymogram development of MMP2 activity from the conditioned media of migrating VSMCs (Fig 5A). Invasion was inhibited by >75% when using the antiserum but was not significantly affected by incubation with an equivalent amount (based on protein concentration) of nonimmune serum (Fig 5B). The antiserum effect was dose dependent (data not shown), and the concentration of antiserum used in these experiments was the minimum dose necessary to achieve the maximal inhibitory effect. There appeared to be a slight inhibition (10%) of migration in the absence of the BM barrier by the antiserum, although the statistical significance of this difference was low (P > .05). Neither the antiserum nor the active MMP peptide affected cell attachment at any time after plating.

**VSMC Invasiveness Is Suppressed by Differentiation**

The VSMCs used in the experiments described above were in the proliferative or synthetic state before plating in the Boyden apparatus. VSMCs can exist in two phenotypic states, a proliferative or synthetic state and a differentiated or contractile state. To determine whether the phenotypic state of VSMCs affects their migratory or invasive ability, differentiated cells were analyzed in the Boyden chamber. Differentiation-specific mRNA expression in proliferating and in vitro differentiated VSMCs was performed to confirm cellular phenotype. High levels of SMHC mRNA and a high ratio of α-smooth muscle to β-cytoplasmic actin mRNA, such as that expressed by intact aortic tissue (lane 1, Fig 6B and 6C), are indicative of smooth muscle–specific differentiation. 49-50 Proliferating VSMCs expressed the β-cytoplasmic isoform of actin (Fig 6B), very low amounts of α-smooth muscle actin (Fig 6A), and little if any SMHC mRNAs (Fig 6C). The in vitro differentiated cells, on the other hand, expressed elevated levels of both α-smooth muscle actin and smooth muscle–specific MHC mRNAs. VSMCs in both states expressed similar amounts of GAPDH and β-actin mRNAs (Fig 6D and 6B, respectively).

VSMC migration across the BM barrier was significantly suppressed by cellular differentiation (Fig 7). Quantitatively, synthetic VSMCs were fourfold to fivefold more active in migrating in the Boyden assay than in vitro differentiated cells. Although the attachment of synthetic/proliferating and differentiated VSMCs to the upper filter of the Boyden apparatus was similar, we performed the following experiment as an additional guarantee that subtle differences in the kinetics of

**Fig 2.** Bar graph of Boyden migration assay illustrating the requirement for a gradient of chemotactic. Invasion assays (migration through an extracellular matrix barrier) were performed as described in “Materials and Methods.” Briefly, Boyden chambers were assembled by adding 10 ng/mL platelet-derived growth factor-BB (PDGF) or 0.1% bovine serum albumin (BSA) to the lower chamber, filters coated with fibronectin/collagen I and supercoated with reconstituted basement membrane were then placed in the apparatus, and 200 000 cells suspended in DMEM containing either 0.1% BSA or 10 ng/mL PDGF were then added to the upper chamber. After incubation at 37°C for 4 hours, cells were fixed on the filter, stained, and counted at ×400 magnification. Filled bar indicates invasion with PDGF in the bottom chamber only; open bar, invasion with no chemotactant; and hatched bar, invasion with PDGF on both sides of the basement membrane barrier.

**Fig 3.** Blots (A) and bar graph (B) showing the effect of matrix metalloproteinase (MMP) propeptides on gelatin zymography and vascular smooth muscle cell (VSMC) migration. A, MMP propeptides inhibit zymogram development of MMPs. Conditioned media from cultured VSMCs were prepared for gelatin zymography as described in “Materials and Methods.” MMP propeptides containing cysteine (active MMP peptide) or serine (inactive MMP peptide) at position 7 were then added at a concentration of 100 μmol/L during renaturation and development of the zymogram. The position of activated (A) and latent (L) forms of the 72-kD gelatinase (MMP2) are indicated by arrows. B, MMP propeptides inhibit invasion but not migration in the absence of a basement membrane barrier. MMP propeptides were added at a final concentration of 100 μmol/L to VSMCs in the upper chamber of the Boyden apparatus during the assays for migration in the presence (invasion) or absence (migration) of a BM barrier. Filled bar indicates the control condition; open bar, serine-containing (inactive) MMP peptide; and hatched bar, cysteine-containing (active) MMP peptide. **P < .0001 (n=18).
adhesion did not affect the results presented above. Both proliferating and differentiated VSMCs were added to the Boyden chamber, in which equimolar concentrations of PDGF were added to the upper and lower chambers. As shown in Fig 2, VSMCs do not migrate to the underside of the filter under this condition. After 4 hours, the attachment of both proliferating and differentiated cells was examined and found to be similar. PDGF in the upper chamber was then removed and replaced with BSA to establish a gradient of chemoattractant. Cells were then allowed to migrate in the presence of this gradient for an additional 4 hours. When analyzed, it was found that the difference between the number of proliferating and differentiated VSMCs that had migrated in this 4-hour period was the same as that shown in Fig 7.

Although the fact that both migration and invasion were inhibited in differentiated cells indicated that some aspect common to both assays was affected, we also looked at whether changes in MMP activity that were important for invasion accompanied the phenotypic changes of VSMCs. Conditioned media from synthetic and differentiated VSMCs were tested for MMP activity using a fluorescent peptide assay. As a positive control, we used recombinant MMP2 that could be activated in vitro. The results presented in Table 2 compare the MMP activity of VSMCs in the two phenotypic states and show that synthetic VSMCs have almost 30 times the activity of differentiated cells. All activity in both VSMC phenotypes and in activated recombinant MMP2 was inhibited by 1,10-phenanthroline (a zinc-chelating inhibitor of metalloproteinase expression) and by the active MMP propeptide used to inhibit migration (Fig 3). In addition, all activity was removed by preabsorption with an affinity-purified MMP2 antibody (Ab 45), indicating that the majority of MMP activity was, in fact, due to MMP2. The data in Table 2 also show that enzyme activity was not altered by PDGF stimulation.

We next looked for possible changes in newly synthesized MMP2 protein (Fig 8) and MMP2 mRNA that could account for the decrease in activity associated

| Table 1. The Effects of Peptide Inhibitors of Metalloproteinases on Recombinant 72-kD Type IV Collagenase Activity and Vascular Smooth Muscle Cell Migration and Invasion |
|---------------------------|------------------|------------------|------------------|
|                          | rhMMP2 Activity (50 ng), relative fluorescence units | Migration % control | Invasion % control |
| Control                  | 1.51±0.07        | ...              | ...              |
| MMP propeptide           |                  |                  |                  |
| Active MMP peptide       | 0.2±0.03*        | 95±6             | 18±3*            |
| Inactive MMP peptide     | 1.43±0.05        | 95±4             | 105±7            |
| Thiol-based inhibitory peptides |
| Phenylalanine derivatives |
| Active SH                 | 0.05±0.01*       | 98±5             | 15±4*            |
| Inactive S-acetate        | 1.52±0.07        | 106±4            | 96±7             |
| Naphthylalanine derivatives |
| Active SH                 | 0.07±0.02*       | 97±6             | 12±4*            |
| Inactive S-acetate        | 1.65±0.05        | 95±7             | 96±8             |

rh indicates recombinant human; MMP, matrix metalloproteinase; and rhMMP2, 72-kD type IV collagenase. Values are mean±SEM.

*P<.0001 compared with untreated control value.

| Table 2. MMP2 Protease Activity in Cultured Vascular Smooth Muscle Cell Media |
|---------------------------|------------------|------------------|------------------|
|                          | Activity, relative fluorescence units |
|                          | Control | 1,10-Phenanthroline | Anti–72-kD MMP Propeptide | Active C7 MMP Propeptide | Inactive S7 MMP Propeptide |
| Recombinant MMP2 (50 ng) | -APMA   | 0.11±0.01          | 0.01±0.01          | ND                  | ND                  |
|                          | +APMA   | 3.1±0.24           | 0.05±0.01          | 0.10±0.02           | 0.05±0.01           | 3.3±0.31            |
| VSMC culture supernatants |
| Proliferative            | 0.85±0.09   | 0.01±0.01          | 0.02±0.01          | 0.01±0.01           | 0.01±0.01           | 0.82±0.08           |
| +PDGF                    | 0.79±0.11   | 0.01±0.01          | ND                  | ND                  | ND                  |
| Differentiated           | 0.03±0.01   | 0.01±0.01          | 0.02±0.01          | 0.01±0.01           | 0.01±0.01           | 0.05±0.01           |
| +PDGF                    | 0.02±0.01   | 0.01±0.01          | ND                  | ND                  | ND                  |
| Media alone              | 0.01±0.01   | ND                  | ND                  | ND                  | ND                  |

MMP indicates matrix metalloproteinase; MMP2, 72-kD type IV collagenase; APMA, p-aminophenylmercuric acetate; VSMC, vascular smooth muscle cell; PDGF, platelet-derived growth factor; and ND, not determined. Values are mean±SEM.
with differentiation. Newly synthesized MMP2 was immunoprecipitated from the conditioned media of metabolically labeled VSMC cultures. The data from three sets of synthetic (lanes 4 through 6) and differentiated (lanes 7 through 9) VSMCs in which the same amounts of conditioned media were immunoprecipitated with the MMP2 affinity-purified antiserum are shown in Fig 8A. The specificity of the immunoprecipitation is shown by the absence of bands in lanes 3 and 5, which are controls.

Fig 5. Blots (A) and bar graph (B) showing that antiserum to the 72-kD type IV collagenase inhibits vascular smooth muscle cell (VSMC) chemoinvasion. A, Anti-72-kD serum inhibits zymogram development of 72-kD type IV collagenase (matrix metalloproteinase 2 [MMP2]). Conditioned media from cultured VSMCs were prepared for gelatin zymography as described in “Materials and Methods.” Nonimmune serum or anti-72-kD serum was added at a 1:50 dilution during renaturation and development of the zymogram. The positions of activated (A) and latent (L) forms of the 72-kD gelatinase (MMP2) are indicated by arrows. B, Anti-72-kD serum inhibits invasion but not migration in the absence of a basement membrane (BM) barrier. MMP propeptides were added at a final dilution of 1:50 to VSMCs in the upper chamber of the Boyden apparatus during the assays for migration in the presence (invasion) or absence (migration) of a BM barrier. Filled bar indicates the control condition; open bar, serine-containing (inactive) MMP peptide; and hatched bar, cysteine-containing (active) MMP peptide. *P > .04 (n = 12); **P < .0001 (n = 12).
Fig 6. Characterization of in vitro differentiated vascular smooth muscle cells (VSMCs). A and B, Northern blotting analysis of mRNA from intact rat aorta, stomach, and brain and from two different cultures of proliferating and differentiated VSMCs. Panel A shows the result of probing the mRNA with the α-smooth muscle actin–specific oligonucleotide; panel B shows the relative levels of α-smooth muscle and β-cytosplasmic actin after hybridization with an actin probe complementary to conserved nucleotide sequences in all actins. C and D, Reverse transcriptase polymerase chain reaction (PCR) analysis of the same mRNA shown in panels A and B. Smooth muscle-specific myosin heavy chain (SmMHC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were measured as described in "Materials and Methods." The positions of the expected sizes of the PCR fragments are indicated to the left of each blot.

by the failure of labeled bands to be precipitated in the absence of the affinity-purified antibody (lane 2) and the absence of bands in which the antibody was first absorbed by the peptide immunogen (lane 3). Although the data show that the amount of radiolabeled MMP2 present in the conditioned media of differentiated VSMCs is greater than the amount in the same volume of media from proliferating cells, the density of differentiated VSMCs is usually five to six times that of proliferating cells. Accordingly, when the data were normalized to cell number, MMP2 protein expression in differentiated VSMCs was only ~60% that in proliferating VSMCs (Fig 8B). The amount of newly synthesized MMP2 protein in both proliferating and differentiated VSMC cultures was unaffected by PDGF-BB (data not shown).

The results presented above are consistent with the zymogram results shown in Fig 4A, which indicate high levels of MMP2 protein expression in both proliferating and differentiated cultures. In this particular zymogram, a fraction of this activity in proliferating cells is in the activated form, whereas the conditioned media from differentiated cells show no activated forms. The presence of activated forms of MMP2 in proliferating cells was not consistently observed, probably because the fraction of zymogram activity in this form was very small. On the other hand, activated forms of MMP2 were never observed in the conditioned media from differentiated cells, unless an activator (e.g., APMA) was added.

Differentiation also reduced the level of MMP2 mRNA approximately in half, while it upregulated mRNA levels of one of the tissue inhibitors of metalloproteinase, TIMP1. The expression of another inhibitor, TIMP2, which has been shown to specifically associate with latent MMP2, was downregulated along with MMP2. The data from a number of such determinations are shown in the bar graph in Fig 9E. All data have been normalized to the expression of GAPDH mRNA, a constitutively expressed housekeeping gene. MMP2 mRNA levels were not affected by the addition of PDGF.

**Discussion**

We show in the present study that isolated VSMCs in cell culture possess the ability to invade a reconstituted BM as they migrate toward the chemoattractant PDGF-BB. Within the time frame of the assay (4 hours), a significant number of the cells adhered to the BM-coated filter and migrated to its underside in the direction of the chemoattractant. The invasive ability of these VSMCs was significantly reduced when MMP activity was inhibited with a peptide based on the conserved region of the pro segment of all MMPs (Fig
by metal-binding thiol-based collagenase-specific peptides (Table 2), or by a neutralizing antibody to MMP2, the 72-kD type IV collagenase (Fig 5). Accordingly, the major MMP expressed in vitro by VSMCs was shown to be MMP2 (Fig 4), which is present in proliferating VSMC-conditioned media in both activated and latent forms. Modulation of VSMCs from a synthetic, proliferating state to a quiescent, differentiated, and contractile phenotype inhibited migration in both the presence and absence of the BM barrier (Fig 6). A common requirement for migration in the presence and absence of the BM barrier is the intracellular transduction of the chemoattractive signal. These results suggest a possible defect in the transduction of the PDGF signal in the differentiated phenotype. Differentiation, however, also resulted in an ≈30-fold reduction in MMP2 activity and an ≈2-fold reduction in MMP2 mRNA accumulation and newly synthesized enzyme, indicating that multiple mechanisms may exist to maintain differentiated VSMCs in a state in which migration is suppressed.

It would be expected that the migration of VSMCs would require, at a minimum, (1) a chemoattractant to direct their movement toward the intima, (2) the ability to breach and traverse the various ECM barriers, and (3) the activation of the cellular machinery for cell movement in response to the chemoattractant. In the in vitro system described here, we used PDGF-BB as a chemoattractant, since considerable evidence supports a role for this growth factor (as well as the PDGF-AB isoform) as a chemoattractant for VSMC migration in vitro and in vivo. These isoforms are likely to be available in the injured vessel, because inhibiting PDGF action with a neutralizing antibody significantly suppresses migration.

![Graph showing MMP Expression and VSMC Migration](image-url)
suppresses VSMC migration and neointimal formation after balloon injury.10

With regard to the second requirement, the ability of cells, such as those responsible for tumor metastasis, to breach ECM barriers is thought to be mediated by proteolytic cascades that may involve the cooperation of serylprotease, cysteinylprotease, and metalloprotease.16,17 Relatively little information about extracellular proteolytic processes in VSMCs exists, with the exception of the role played by the serine protease plasmin. Plasmin is generated by VSMCs from plasminogen through the action of UPA and TPA.18 Plasmin not only degrades fibrin but is also capable of digesting several ECM glycoproteins and activating many procollagenases by proteolytic processing, converting them from the latent to the active form.19 Plasmin-dependent pathways of ECM degradation may play an important role in VSMC migration. In tumor cells, inhibitors of plasmin-dependent proteolysis significantly decrease metastasis.16,19 In the rat carotid artery, the expression of TPA is increased in the medial area below the site of injury over a time course consistent with VSMC migration.20 Furthermore, the well-documented effectiveness of heparin and heparan sulfate proteoglycans in inhibiting neointimal proliferation after balloon angioplasty of the rat carotid artery51,52 may be primarily due to their effects on reducing VSMC migration by inhibiting TPA gene expression.53

Recent observations indicate that VSMCs possess both plasmin-dependent and plasmin-independent pathways for ECM degradation.14 Our results show that one of the possible plasmin-independent pathways for ECM remodeling involves MMPs, in particular, MMP2, the 72-kd type IV collagenase. The specificity of MMP2 action toward type IV collagen is consistent with its role in BM degradation. Human and primate VSMCs have also been shown to constitutively express MMP2 in culture.54 Given the dramatic effects that BM-type ECMs have on VSMC differentiation42-56 and the fact that a BM surrounds each VSMC in the tunica media,13 the degradation of this matrix may be important not only in removing a structural barrier to migration but also as an important initial step in the phenotypic transformation VSMCs undergo during vessel disease or injury.

It has recently been reported that both MMP2 and MMP9, the 72- and 92-kd type IV collagenases (gelatinases), are present in commercially available reconstituted BM preparations.48 We believe that the presence of these MMPs in the BM does not significantly affect our results. This belief is based on the following quantitative consideration. According to a recent report,48 10 µg BM (the amount used per filter) contains ~0.03 and 0.06 ng of MMP2 and MMP9, respectively. Since a known amount of recombinant MMP2 was included as a positive control in every assay that was performed, we can make an approximate estimate of the absolute amount of MMP2 activity produced by our VSMC cultures. That estimate is 1.6 to 2.4 ng. Even if all of the residual MMP2 and MMP9 in the BM preparation were active, our cells are still producing 50 to 75 times the amount of MMP2 activity contributed by the BM. Although MMP9 has been shown to be important in trophoblast invasion of BM in the uterine wall,54 our studies show that virtually all of the MMP activity in the conditioned media of VSMCs is MMP2 (Table 2). In addition, we have used an antisera to MMP9 (gift of W. Stetler-Stevenson, National Cancer Institute, Bethesda, Md) that inhibits the development of 92-kd gelatinase activity in azymogram and found it to have no effect on invasion (data not shown). These observations suggest that MMP9 does not play a significant role in VSMC invasion under the conditions of our experiments.

Since VSMCs are also embedded in an interstitial matrix that contains collagen type I as a principal structural ingredient, it would be expected that VSMCs express and/or regulate interstitial collagenase (MMP1) activity. Expression of latent or pro-MMP1 has been detected in human VSMC cultures stimulated with either PDGF or PMA.20 The stimulation by PDGF was, in fact, found only in VSMCs obtained from older individuals (>54 years), whereas PMA stimulated MMP1 expression in cultures from both young and old individuals. However, we have not detected MMP1 by either zymography or by Northern blotting (Fig 4) in the VSMCs used in the present study, even when the cultures were treated with PMA (data not shown). Cytokines, such as interleukin-1 and tumor necrosis factor-α, are potent physiological stimuli for MMP1 expression that are expressed by, at least, some VSMC cultures.55,56 The expression of these cytokines may be an important permissive requirement for MMP1 expression and may be lacking in our system. On the other hand, the lack of rat VSMC responsiveness may represent an important species difference that must be taken into account in extrapolating the results reported here to human VSMCs and vascular disease states.

The most significant and novel observation reported here is that the invasive phenotype is suppressed by VSMC differentiation. In vivo and in cell culture, VSMCs can exist in different phenotypic states with respect to cellular differentiation.49,50 In the normal intact vessel, VSMCs exist primarily in a differentiated state in which gene expression is directed toward the synthesis of differentiation-specific products, such as contractile proteins. In response to injury, the VSMCs that accumulate in extramedial spaces dedifferentiate toward a synthetic state in which gene expression is directed toward cell growth and proliferation.57 Modulation between these two states can be readily achieved in cell culture by altering cell density and the availability of growth factors.49,60 At low cell density and in the presence of serum growth factors, VSMCs exist in a dedifferentiated state similar to that seen in proliferating VSMCs from the neointima after vessel injury. Allowing VSMCs in cell culture to grow past confluence to a postconfluent state in which they attain a three-dimensional hill-and-valley organization and then withdrawing serum growth factors for a number of days results in a gradual phenotypic transformation toward the differentiated state. Our results demonstrate that VSMC differentiation suppresses the invasive phenotype. The regulation of invasive ability in VSMCs by cell differentiation is one important difference between the invasive behavior of VSMCs and tumor cells.

One way in which the suppression of invasive ability might be achieved is through a reduction in MMP2 activity (Table 2). That reduction can result from changes at a number of different levels of regulation. We have observed a number of differences in the
processing of MMP2 and expression of endogenous inhibitors of the MMP that could account for this change. Differentiated cells express less MMP2 mRNA (Fig 9), which is reflected in the amount of newly synthesized MMP2 protein secreted into the culture media (Fig 8). Differentiation also resulted in elevated levels of the endogenous tissue inhibitor of metalloproteinases, TIMP1. Since the relative levels of MMP2 and the specific inhibitors of the MMPs, the TIMPs, ultimately determine the level of net collagenolysis, the effect of a decrease in MMP2 protein coupled with an increase in TIMP1 is likely to lead to less active MMP2. Interestingly, the level of TIMP2 decreased along with MMP2. Unlike TIMP1, however, TIMP2 binds to both active and latent MMP2.58 Binding to the latent proenzyme occurs at a site different from that in the active enzyme or the site at which TIMP1 binds, suggesting that TIMP2 may have a function distinct from inhibiting metalloproteinase activity. The TIMPs have homology to some cytokines and have been shown to inhibit cell proliferation.17,41,59

In addition to suppressing MMP2 activity, there is likely to be a second mechanism to suppress the invasive ability of the VSMCs. That is because migration in the presence and absence of the BM barrier was inhibited by cellular differentiation. Inhibiting MMP activity has no effect on migration in the absence of the BM barrier (Fig 3 and 5, Table 1), so some feature common to both types of migration must also be altered by differentiation. Of the many possibilities, alterations in chemotactic signal transduction or the regulation of actin microfilament assembly and disassembly required for cell movements seem probable targets of the suppression.

The development and application of the in vitro assay described here will aid in the identification of the factors responsible for determining when and how the invasive phenotype is acquired by VSMCs. The reduction in in vitro invasive ability after VSMC differentiation might be one of the mechanisms that limits cell migration and ECM degradation by VSMCs in the normal vessel and restricts these phenomena to periods after vessel injury.

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