Type VIII Collagen Stimulates Smooth Muscle Cell Migration and Matrix Metalloproteinase Synthesis after Arterial Injury

Guangpei Hou, Diane Mulholland,
Margaret Anna Gronski, and
Michelle Patricia Bendeck

From the Terrence Donnelly Research Laboratories, Division of Cardiology, St. Michael's Hospital, and the Departments of Medicine and Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

Type VIII collagen is a matrix protein expressed in a number of tissues undergoing active remodeling, including injured arteries during neointimal formation and in human atherosclerotic plaques; however, very little is known about its function. We have investigated whether the type VIII collagen stimulates smooth muscle cell (SMC) migration and invasion by binding to integrin receptors and up-regulating matrix metalloproteinase (MMP) production. SMCs attached to plates coated with type VIII collagen in a dose-dependent manner, with maximal attachment occurring with coating solutions containing 25 $\mu\text{g}/\text{ml}$ collagen. Type VIII collagen at 100 $\mu\text{g}/\text{ml}$ stimulated an 83-fold increase in the migration of SMCs in a chemotaxis chamber. Antibodies against $\beta 1$ integrin receptors prevented attachment and migration of SMCs. Antibodies against $\alpha 1$ or $\alpha 2$ integrins reduced attachment of SMCs to type VIII collagen by 29% and 77%, respectively. We found that SMCs grown from the rat neointima, but not medial SMCs, increased their production of MMP-2 and -9 on adherence to type VIII collagen. This suggests that there is an important difference in phenotype between intimal and medial SMCs and that intimal SMCs have distinct matrix-dependent signaling mechanisms. Our findings suggest that type VIII collagen deposited in vascular lesions functions to promote SMC attachment and chemotaxis, and signals through integrin receptors to stimulate MMP synthesis, all of which are important mechanisms used in cell migration and invasion. (*Am J Pathol* 2000, 156:467–476)

Smooth muscle cells (SMCs) contribute to atherosclerosis and restenosis by proliferation, migration from media to intima, and deposition of an abundant extracellular matrix in the neointima. Recent research suggests that the extracellular matrix is not simply an inert scaffold, but

instead that there are dynamic interactions between cells and matrix, which contribute to SMC responses after injury. In fact, several matrix proteins produced in abundance after arterial injury stimulate SMC proliferation and/or migration *in vitro*, including osteopontin, fibronectin, tenascin, hyaluronic acid, and type I collagen.^{1–7} Cell migration is a complex process stimulated by chemotaxis (directed movement along a gradient of a chemical stimulus). Cells must attach to a matrix to gain traction for migration, but they also have to detach from the matrix to translocate. Attachment and release are precisely coordinated by focal localization of matrix-degrading proteinases on the cell surface, and matrix metalloproteinases (MMPs) also facilitate cell invasion by clearing a path through complex, three-dimensional extracellular matrices.⁸

In the current study, we investigate the role of type VIII collagen in SMC migration. Type VIII collagen is a short-chain collagen expressed during active tissue remodeling, including angiogenesis, embryonic development of the heart, and glomerulonephritis.^{9–11} We showed dramatic up-regulation of type VIII collagen expression by SMCs, using differential display screening to identify genes that were overexpressed in the rat carotid artery after injury.¹² The protein was present only transiently, during the first 7 days after balloon catheter injury, in coincidence with early SMC migration. Furthermore, when we inhibited SMC migration by treating rats with antibodies against platelet-derived growth factor-BB and fibroblast growth factor-2, both strong chemotactic stimuli, messenger RNA (mRNA) expression for type VIII collagen was reduced in correlation with migration. Subsequent studies confirmed our observations of type VIII collagen expression after rat carotid injury¹³ and in the balloon-injured iliacs of cholesterol-fed rabbits.¹⁴ The potential relevance to human vascular disease was suggested by expression in atherosclerotic plaques, but not in normal media of coronary arteries.¹⁵ Taken together, these studies suggest an important role in cell migration

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Address reprint requests to Dr. Michelle P. Bendeck, Vascular Biology Research, Rm 8–013 Queen, St. Michael's Hospital, 30 Bond St., Toronto, Ontario, Canada M5B 1W8. E-mail: bendeckm@smh.toronto.on.ca.

during vascular remodeling; however, there is still little concrete evidence of type VIII collagen function.

Type VIII collagen expression in the injured rat carotid¹² was coincident with early and dramatic increases in MMP-2 and MMP-9 expression and activity, which are critical for SMC migration from the media to the intima.^{16–19} Very recently, reports have been published co-localizing type VIII collagen and MMP-1 expression in SMCs in the intima of injured iliac arteries¹⁴ and in macrophages of the atherosclerotic plaque.²⁰ Taken together, these data suggest important parallels in type VIII collagen and MMP expression; however, it is not clear whether the two are directly related. In several other cell types, matrix components can stimulate MMPs,^{21–29} but this has never been investigated in SMCs.

When an artery is injured, SMCs within the media of the vessel undergo a switch from a quiescent to an active phenotype, which is characterized by proliferation, migration, and synthesis of extracellular matrix. SMCs that have migrated to the intima of an injured vessel maintain many aspects of this active phenotype, even when the cells are isolated and grown in tissue culture.³⁰ This indicates that phenotypic dedifferentiation is not solely a function of the local environment. Intimal SMCs grow faster in culture, are more responsive to growth factors, and express a wide array of genes that are not expressed or expressed at very low levels by SMCs derived from the quiescent media of an uninjured vessel.³¹ In the current study, we have taken advantage of the ability to isolate and grow in culture SMCs derived from the media of an uninjured, quiescent rat carotid (medial SMCs) and activated SMCs derived from the intima of the injured rat carotid (intimal SMCs). We have compared interactions of these two types of SMC with type VIII collagen, because the responses of the different cell phenotypes may be very important in the pathogenesis of diseases such as atherosclerosis.

In this study we investigate the hypothesis that type VIII collagen promotes SMC migration, using *in vitro* models to mimic critical steps of the migration process. Type VIII collagen stimulates SMC attachment, focal adhesion formation, and chemotaxis of medial and intimal SMCs. These effects are mediated via $\alpha 2\beta 1$ and $\alpha 1\beta 1$ integrin receptors. We also show that type VIII collagen stimulates MMP-2 and MMP-9 expression and activity in intimal, but not medial, SMCs. These studies suggest that type VIII collagen plays a critical role in regulating SMC invasion and migration.

Materials and Methods

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Smooth Muscle Cell Culture

Male Sprague-Dawley rats were obtained from Charles River (Montreal, PQ, Canada). Uninjured carotid arteries were harvested and stripped of adventitia and the endothelium was scraped off, then medial SMCs were dispersed by digestion for 1 hour in 0.3 mg/ml elastase type

III, 1.8 mg/ml collagenase type I (Worthington, Freehold, NJ), 0.44 mg/ml soybean trypsin inhibitor, 2 mg/ml bovine serum albumin (BSA).¹ To obtain intimal SMCs, left carotid arteries of rats were injured with a balloon catheter, and, 2 weeks later, the thickened neointima was stripped from the vessel with the aid of a dissecting microscope.³⁰ Intimal SMCs were dispersed by digestion with elastase and collagenase as described above. Six carotids were pooled for isolation of medial SMCs, and six intimas were pooled for isolation of intimal SMCs. In addition, to ensure consistency of the SMC phenotypes across various different rats, we obtained and maintained several independent dispersions, which were randomly selected for experiments. Intimal and medial SMCs were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2% penicillin-streptomycin and used between passages 5 and 10. Immunostaining for smooth muscle α -actin confirmed that the harvested cells were SMCs. DMEM, penicillin-streptomycin, trypsin, FCS, and fibroblast growth factor-2 were from Life Technologies, Inc. (Gaithersburg, MD).

Human newborn aortic smooth muscle cells (HF16) were generously provided by Dr. Cecilia Giachelli of the University of Washington (Seattle, WA).

SMC Attachment Assay

Type VIII collagen was purified from bovine Descemet's membrane as previously described.³² To summarize, bovine eyes were obtained from a local slaughterhouse and corneas were dissected from the eyeballs, then the inner Descemet's membranes were peeled off with forceps. The membranes were digested with 0.5 mg/ml pepsin in 0.5 mol/L acetic acid for 12 hours and centrifuged, and the supernatant was lyophilized then resolubilized in 1 mol/L NaCl, 50 mmol/L Tris, pH 7.5. The collagens were separated from other proteins by a series of precipitations in NaCl (4 mol/L, 0.7 mol/L and 1.5 mol/L), each followed by dialysis against 0.5 mol/L acetic acid. Final separation of type VIII collagen from contaminating type V collagen was achieved by chromatography on an agarose A1.5-m column (BioRad, Hercules, CA). Purity of the preparation was confirmed by analysis of Coomassie-blue stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of the protein (one band evident at 50 kd), and Western blots probed with an antibody against bovine type VIII collagen also showed a single band at 50 kd.

Pepsin-solubilized bovine dermal type I collagen Vitrogen 100 (Collagen Biomaterials) was used as a positive control substrate based on previous reports of its ability to promote SMC attachment.^{6,33,34} Type VIII or type I collagen was diluted in phosphate-buffered saline (PBS) to the stated concentrations, then 50 μ l per well was added to 96-well tissue culture plates and incubated overnight at 4°C. Coating efficiency was determined by iodinating type I and type VIII collagen (Iodogen, Pierce, Rockford, IL) and measuring the amount of protein coated as a percentage of total counts applied. Coating

efficiency did not vary over the range of substrate concentrations applied; it was similar for both 10 $\mu\text{g/ml}$ (37.1%) and 100 $\mu\text{g/ml}$ (33.5%) type I collagen applied to wells. Coating efficiency was also similar for 10 $\mu\text{g/ml}$ (27.4%) and 100 $\mu\text{g/ml}$ (29.9%) type VIII collagen applied to wells. For attachment assays, after coating overnight, the wells were rinsed with PBS, and nonspecific binding sites were blocked with 10 mg/ml BSA at 37°C for 1 hour. SMCs were detached from culture flasks by minimal trypsinization (1–2 minutes in 0.05% trypsin), placed immediately into an equal volume of 0.5 mg/ml soybean trypsin inhibitor and centrifuged. Cells were resuspended in DMEM with 1 mg/ml BSA and counted using a hemocytometer. Cells (30,000) were plated in each well and allowed to attach at 37°C for 60 to 90 minutes. Nonadherent cells were rinsed off with PBS, and the remaining attached cells were fixed with 4% paraformaldehyde for 5 minutes, then stained for 5 minutes with 0.5% toluidine blue dissolved in 4% paraformaldehyde and rinsed in water. Attached, stained cells were solubilized with the addition of 100 μl of 1% sodium dodecyl sulfate, and absorbance was measured in a microtiter plate reader (Molecular Devices) at 595 nm. The validity of this measure of attachment was assessed by plotting toluidine blue absorption against cell counts, which were derived by trypsinizing to release cells from the wells and counting them with a Coulter counter. An excellent correlation ($r^2 = 0.986$) between the two techniques was achieved. Experiments were performed in triplicate and repeated a minimum of three times. Attachment assays were performed using rat medial or intimal SMCs or human HF16 SMCs.

Vinculin Immunostaining

Glass coverslips placed in 24-well culture plates (Costar, Corning, NY) were coated with 10 $\mu\text{g/ml}$ type VIII collagen. Medial SMCs (80,000) suspended in 1 mg/ml BSA/DMEM were added to each well and allowed to adhere for 2 or 4 hours, after which the coverslips were washed with PBS to remove nonadherent cells, and remaining cells were fixed in 4% paraformaldehyde for 7 minutes. The cells were treated with 0.2% Triton X-100 to block nonspecific binding, then stained with anti-human vinculin antibody at 1:600 dilution for 30 minutes, and then Cy3-conjugated anti-mouse immunoglobulin G (IgG) at 1:200 dilution (Jackson ImmunoResearch, West Grove, PA). Fixing, blocking, and staining steps were followed by three 5-minute washes in PBS. The coverslips were mounted on glass slides with a 1:1 solution of PBS:glycerol and observed under a fluorescent microscope.

Integrin Receptor Blocking

Receptors for type VIII collagen were determined with blocking antibodies against integrin receptors to inhibit attachment or focal adhesion formation. Rat medial or intimal SMCs were incubated with anti-rat integrin $\beta 1$ antibody (clone Ha2/5, Pharmingen, San Diego, CA) or anti-rat integrin $\beta 3$ antibody (clone F11, Pharmingen) at a

concentration of 20 $\mu\text{g/ml}$ for 30 minutes at room temperature before plating for attachment or focal adhesion assays on 10 $\mu\text{g/ml}$ type VIII collagen in the presence of antibody.

Human SMCs were used to determine the α integrin subunit for type VIII collagen, because there are few blocking antibodies available for rat α integrin subunits. Experiments were performed as described above for rat SMCs. Blocking antibodies against human integrins were obtained from Chemicon and included $\alpha 1$ (FB12), $\alpha 2$ (P1E6), $\alpha 3$ (P1B5), $\alpha 4$ (P1H4), $\alpha 5$ (P1D6), $\alpha 6$ (CLB701), αv (P3G8), $\beta 1$ (6S6), and $\alpha v\beta 3$ (LM609).

SMC Migration Assay

Type VIII or type I collagen was diluted to indicated concentrations in DMEM containing 200 $\mu\text{g/ml}$ BSA and placed in the bottom wells of 24-well transwell chemotaxis chambers (Costar). Freshly trypsinized rat SMCs were washed twice in soybean trypsin inhibitor, then resuspended in DMEM containing 200 $\mu\text{g/ml}$ BSA. Cells (50,000) were plated on polycarbonate filters with 8- μm pores, inserted into the wells containing chemoattractants, and allowed to migrate for 4 hours at 37°C in a humidified chamber. After the incubation period, the tops of the filters were scrubbed with a cotton swab to remove cells, and the cells that had migrated to the bottom of the filter were fixed and stained with Diff-Quick fixative and staining solutions (Dade Diagnostics). The filters were cut from the wells and mounted under oil on glass slides. Migration was quantitated by counting the number of cells in five random 200 $\times$ fields/filter and expressed as the average number of cells per field. Counts were performed using a digital image analysis system (Simple, C-Imaging Systems). Each experiment was performed in triplicate and repeated three times. To differentiate chemotaxis from chemokinesis, we performed checkerboard assays. Type VIII or type I collagen, at concentrations ranging from 0 to 100 $\mu\text{g/ml}$, was dissolved in media in both the top and bottom chambers of the well or in the top chamber only. Under these conditions, in the absence of a chemotactic gradient, SMC migration is due to random chemokinesis.

Blocking antibodies against integrin receptors were used to inhibit migration toward type VIII collagen. SMCs were incubated with anti-rat integrin $\beta 1$ antibody (clone HA2/5, Pharmingen) or anti-rat integrin $\beta 3$ antibody (clone F11, Pharmingen) at a concentration of 20 $\mu\text{g/ml}$ for 30 minutes at room temperature before plating SMCs in wells and performing migration assays in the presence of the blocking antibodies.

MMP Activity Measurement

MMP activity was measured using gelatin zymography. Intimal or medial SMCs were suspended in serum-free DMEM containing 200 $\mu\text{g/ml}$ BSA and plated in 96-well plates coated with type VIII collagen. After 24 hours, conditioned medium was collected from the wells and assayed for MMP activity as we have previously de-

scribed.¹⁶ Briefly, conditioned medium samples were subject to electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels that contained 0.1% gelatin as a substrate for MMP digestion. After electrophoresis, the gels were incubated 16 hours and then stained with Coomassie blue (BioRad), and MMP activity was evident as cleared bands of substrate lysis. The MMPs were identified by their molecular weights and inhibition by ethylenediaminetetraacetic acid or phenanthroline.

Northern Blots for MMP-2 and MMP-9 mRNA Expression

Rat MMP-2 and MMP-9 complementary DNA (cDNA) probes were cloned by reverse transcriptase-polymerase chain reaction of RNA extracted from rat intimal SMCs. The primers for MMP-2 were as follows: sense, 5'-TTT GAT GAC GAT GAG CTA-3'; antisense, 5'-GGG AGC TCA GGC CAG AA-3'. Total length of probe 932 bp.³⁵ Primers for MMP-9 were as follows: sense, 5'-GAT GGT TAT CGC TGG TGC GCC-3'; antisense, 5'-GTG CAG TGG AAC ACA TAG TGG-3'. Total length of this probe was 496 bp.³⁶ Reverse transcription was performed using the Advantage 1st-strand cDNA synthesis kit by the manufacturer's instructions (Clontech, Palo Alto, CA). To summarize, 0.2 μ g of mRNA was heat denatured in diethyl pyrocarbonate-treated water for 2 minutes at 70°C and then incubated at 42°C for 1 hour in a total reaction volume of 20 μ l containing 20 pmol of primer (oligodeoxythymidylic acid¹⁸), 0.5 mmol/L of each deoxynucleoside triphosphate, 1 U/ μ l of RNase inhibitor, and 200 U of Moloney murine leukemia virus reverse transcriptase. The reaction was stopped by heating at 94°C for 5 minutes, the mixture was diluted to a final volume of 100 μ l, and aliquots were stored at -80°C until further use. PCR amplification of specific cDNA was achieved using 2.5 U of *Taq* DNA polymerase and 0.2 μ mol/L primers in 100 μ l of reaction mixture. Polymerase chain reaction cycling was carried out as follows: denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 1 minute for 30 cycles.

Intimal SMCs were plated in wells precoated with type VIII collagen at concentrations ranging from 5 to 100 μ g/ml, and incubated for 24 hours. Total cellular RNA was isolated, and Northern blots were prepared by our previously published methods.¹² The cDNA probes for rat MMP-2 and MMP-9 were labeled with ³²P-labeled deoxycytidine triphosphate, using a Multiprime kit (Amersham). The hybridized blots were used to expose Hyperfilm-MP (Amersham).

Statistical Analysis

Attachment and migration assay results were analyzed by analysis of variance followed by Fisher's protected least significant difference.

Figure 1. Adhesion of medial SMCs (A) or intimal SMCs (B) to type VIII collagen (●) or type I collagen (○). Plates were coated with the indicated concentrations of substrates. Adhesion was quantitated by toluidine blue staining; the absorbance of toluidine blue at 595 nm is proportional to the number of adherent cells. Values are mean $\pm$ SEM. *Attachment to type VIII collagen was significantly less than attachment to type I collagen at equivalent concentrations.

Results

Type VIII Collagen Promotes Attachment of SMCs

Medial SMCs attached to wells coated with type VIII or type I collagen in a dose-dependent manner (Figure 1A). Maximal attachment occurred at 25 μ g/ml type VIII collagen, with an optical density of 0.189 ± 0.010 , or 100 μ g/ml type I collagen, with an optical density of 0.386 ± 0.018 . Attachment to type VIII collagen was significantly less than attachment to type I collagen at all concentrations tested. The molecular mass of type I collagen is ~95 kd, and the molecular mass of type VIII collagen isolated by pepsin digestion from Descemet's membrane is ~50 kd. When compared on an equimolar basis, medial SMC attachment to type VIII collagen was still significantly less than attachment to type I collagen. For example, when plates were coated with a 1 mmol/L con-

Figure 2. Photomicrograph of medial SMCs incubated on coverslips coated with 10 µg/ml type VIII collagen, then stained with an antibody against vinculin to localize focal adhesions. **A:** 2-hour incubation. **B:** 4-hour incubation. **C:** 4-hour incubation in presence of normal mouse IgG. **D:** 4-hour incubation in the presence of anti-β1 integrin antibody. Original magnification, ×1000.

centration of collagen, attachment to type VIII collagen was 0.192 ± 0.022 , whereas attachment to type I collagen was 0.386 ± 0.018 .

Intimal SMCs attached to type VIII or type I collagen-coated wells in a dose-dependent manner (Figure 1B). Attachment of intimal SMCs at low concentrations of type VIII collagen (5–20 µg/ml) was equivalent to attachment to type I collagen at these concentrations. However, at higher concentrations (25–100 µg/ml), attachment of intimal cells to type VIII collagen was significantly less than attachment to type I collagen. When compared on an equimolar basis, at higher concentrations, intimal SMC attachment to type VIII collagen was significantly less than attachment to type I collagen. For example, when plates were coated with a 1 mmol/L concentration of collagen, attachment to type VIII collagen was 0.252 ± 0.024 , while attachment to type I collagen was 0.395 ± 0.010 .

SMCs Form Focal Adhesions on Type VIII Collagen

Assembly of focal adhesion complexes occurs after cell surface integrins bind to the extracellular matrix and ag-

gregate, initiating reorganization of the cytoskeleton and a cascade of cell-signaling events. To determine whether SMC binding to type VIII collagen was mediated through integrins and focal adhesions, medial SMCs plated on type VIII collagen were stained with an antibody against vinculin, which is a cytoskeletal component of the focal adhesion complex. Vinculin staining was localized at discrete focal adhesions 2 hours after plating on 10 µg/ml type VIII collagen (Figure 2A), and by 4 hours the cells were well spread and had formed numerous distinct focal adhesions (Figure 2B). Similar results were obtained with intimal SMCs (data not shown). No cell adhesion or spreading was observed in control wells coated with only BSA.

The principle β integrin subunits expressed on mesenchymal cells are β1 and β3 integrin receptors; therefore, we used adhesion-blocking monoclonal antibodies against these integrin subunits to determine the receptor mediating interactions between SMCs and type VIII collagen. Pretreatment of medial SMCs with 20 µg/ml of a monoclonal antibody against rat β1 integrin prevented SMC focal adhesion formation on type VIII collagen (Figure 2D), whereas normal mouse IgG had no effect (Figure 2C).

Figure 3. Attachment of medial SMCs (A) or intimal SMCs (B) to type VIII collagen. Cells were preincubated with normal mouse IgG (NlgG), anti-β3 integrin antibody (anti-β3), or anti-β1 integrin antibody (anti-β1) for 30 minutes before plating on wells precoated with 10 μg/ml type VIII collagen. Cells were allowed to adhere for 90 minutes, and then attachment was quantitated by toluidine blue staining as described in Materials and Methods. Values are mean ± SEM. *Value is significantly different from NlgG.

α2β1 and α1β1 Integrin Receptors Mediate SMC Binding to Type VIII Collagen

Blocking antibodies to various integrin receptors were used to determine which α and β subunit pair(s) bind to type VIII collagen. Pretreatment of medial SMCs with 20 μg/ml of an antibody against rat β1 integrin reduced attachment to 10 μg/ml type VIII collagen by 92% in comparison with normal mouse IgG-treated controls (Figure 3A). By contrast, pretreatment with an antibody against rat β3 integrin (20 μg/ml) had no significant effect (Figure 3A). The results were similar for intimal SMCs; the anti-β1 antibody reduced intimal SMC attachment to type VIII collagen by 83%, whereas anti-β3 had no effect (Figure 3B). For these blocking assays, SMCs were plated in wells coated with 10 μg/ml type VIII collagen, a concentration that gave half maximal attachment.

Because blocking antibodies against the rat α integrin subunits were not available, experiments were performed using a human SMC strain, HF16, to identify the α subunit(s) that binds to type VIII collagen. HF16 SMC attachment to type VIII collagen was maximal at a coating concentration of 20 μg/ml, with an optical density of 0.314 ± 0.032 . Pretreatment with an antibody against

Figure 4. Inhibition of attachment of human HF16 smooth muscle cells to type VIII collagen with integrin-blocking antibodies. Cells were preincubated with normal mouse IgG (NlgG), or anti-α1 integrin, anti-α2 integrin, or anti-β1 integrin antibody for 30 minutes before plating on wells precoated with 20 μg/ml type VIII collagen. Cells were allowed to adhere for 90 minutes, and then attachment was quantitated by toluidine blue staining as described in Materials and Methods. Values are mean ± SEM. *Value is significantly different from NlgG.

human β1 integrin (20 μg/ml) reduced attachment by 97% (Figure 4). Blocking antibodies against α2 or α1 integrins reduced adhesion to type VIII collagen by 77% and 29%, respectively (Figure 4). By contrast, antibodies against human α3, α4, α5, α6, αv, and αvβ3 did not affect attachment to type VIII collagen (data not shown). Pretreatment with soluble GRGDSP peptides (Life Technologies) did not affect adhesion to type VIII collagen, which suggested that binding was not mediated through RGD sites (data not shown).

Type VIII Collagen Stimulates SMC Migration

We tested for a chemotactic effect of type VIII collagen on SMC migration, using modified Boyden chambers. Type VIII collagen stimulated the dose-dependent, directed migration of rat medial SMCs, with maximal migration of 126.6 ± 32.1 cells/field occurring at a concentration of 100 μg/ml (Figure 5A). The chemokinetic effect of type VIII collagen was assessed using a checkerboard assay, in which the chemotactic gradient was abolished by adding equal concentrations of protein to both top and bottom wells of the chemotaxis chamber or to the top well of the chamber only. Cell movement due to chemokinesis was very low; less than five cells per field migrated through the filter when 100 μg/ml type VIII collagen was added to both wells or to the top well only. Type I collagen was used as a positive control for the assay, because it is a known SMC chemotactic factor.⁷ At equivalent concentrations of collagen, chemotaxis toward type VIII collagen was not significantly different from chemotaxis toward type I collagen (Figure 5A). Also, comparing equimolar concentrations of collagen, there were no significant differences between migration toward either substrate. Dose-dependent increases in intimal SMC migra-

Figure 5. A: Migration of medial SMCs toward type VIII collagen (●) or type I collagen (○). Type VIII or type I collagen was dissolved in the bottom of a chemotaxis chamber at the indicated concentrations, and cells were plated on nylon filters with pores 8 μm in diameter. Migration was measured by counting cells that migrated to the bottom of the filter and was expressed as cells per field at $\times 200$ magnification. Values are mean $\pm$ SEM. **B:** Inhibition of migration of medial SMCs toward 50 $\mu\text{g/ml}$ type VIII collagen after treatment with integrin antibodies. Medial SMCs were preincubated with normal mouse IgG (NIgG), anti- $\beta 3$ integrin antibody (anti- $\beta 3$), or anti- $\beta 1$ integrin antibody (anti- $\beta 1$) for 30 minutes before plating. Values are mean $\pm$ SEM. *Value is significantly different from NIgG.

tion toward type VIII collagen were similar to medial SMCs (data not shown).

To determine the integrin receptor mediating migration toward type VIII collagen, cells were pretreated with blocking antibodies to rat $\beta 1$ and $\beta 3$ integrins, and chemotaxis assays were done in the presence of antibodies. Preincubating medial SMCs with an anti- $\beta 1$ integrin antibody (20 $\mu\text{g/ml}$) almost completely abolished migration toward 50 $\mu\text{g/ml}$ type VIII collagen; there was a 97% reduction compared with cells incubated with normal mouse IgG (Figure 5B). By contrast, anti- $\beta 3$ integrin antibody did not inhibit type VIII collagen-stimulated migration.

Attachment to Type VIII Collagen Stimulates Intimal SMC, but Not Medial SMC Gelatinase Expression and Activity

Production of MMPs is another important step for SMC migration and invasion through three-dimensional matrices, thus we tested the hypotheses that type VIII or type

Figure 6. A: Gelatin zymograms with conditioned media from rat medial (top) and intimal (bottom) SMCs plated on different concentrations of type VIII collagen as indicated and incubated for 24 hours. Medial SMCs produced two bands with molecular masses of 72 and 70 kd; these represent latent MMP-2 (MMP-2 L). Intimal SMC produced bands at 72 and 70 kd (MMP-2 L), 62 kd representing active MMP-2 (MMP-2 A), and 88 kd representing active MMP-9 (MMP-9 A). **B:** Northern blots with total cellular RNA from rat intimal SMCs plated on different concentrations of type VIII collagen as indicated. The blot was probed with cDNAs for MMP-9 (top) and MMP-2 (middle). The ethidium bromide-stained gel with 28S and 18S ribosomal bands is shown (bottom) to demonstrate equal loading of RNA.

I collagen stimulates MMP expression and activity. SMCs were plated on collagen, and MMP activity released into the culture media was assayed using gelatin zymograms. Medial SMCs plated on type VIII collagen produced two prominent bands of gelatinolytic activity with molecular masses of 72 and 70 kd (Figure 6A). There was no change in activity in these bands with increasing concentration of type VIII collagen. Intimal SMCs plated on 5 $\mu\text{g/ml}$ type VIII collagen also produced two bands with molecular masses of 72 and 70 kd. Activity in the 72- and 70-kd bands was increased in a dose-dependent manner with increasing type VIII collagen concentration (Figure 6A). In addition, two new bands with molecular masses of 62 and 88 kd were induced in a dose-dependent manner in the intimal SMCs. Based on molecular masses and comparison with previous reports including our own,

these bands likely represent the following MMPs: latent zymogen forms of MMP-2 (72- and 70-kd bands; MMP zymogens appear active on the zymogram because SDS in the gel partially denatures the enzyme, exposing the active site); MMP-2 active enzyme (62 kd); and MMP-9 active enzyme (88 kd).¹⁶ Medial or intimal SMCs plated on type I collagen produced only the latent MMP-2 bands, and there was no relationship between MMP production and type I collagen concentration (data not shown).

To determine whether the changes in MMP activity in intimal SMCs plated on type VIII collagen were a result of increased steady-state levels of mRNA, we prepared Northern blots containing total cellular RNA extracted from intimal SMCs, and probed these with cDNAs to MMP-2 and MMP-9 (Figure 6B). Both MMP-2 and MMP-9 mRNA expression were increased after plating intimal SMCs on increasing concentrations of type VIII collagen ranging from 5 to 100 $\mu\text{g/ml}$ (Figure 6B). Bands of 3.1 kb and 3.6 kb were detected on the blots, corresponding to mRNA transcripts for MMP-2 and MMP-9, respectively.¹⁶ Thus the change in mRNA levels for the two MMPs paralleled the change in activity, suggesting that increased production was mediated at least in part through transcription.

Discussion

The results presented here clearly show that type VIII collagen promotes SMC attachment and chemotaxis. Furthermore, type VIII collagen stimulates MMP-2 and MMP-9 synthesis by SMCs, specifically by activated intimal SMCs. Taken together with several descriptive studies that have documented type VIII collagen production in coincidence with migration at sites of arterial injury, these results suggest that type VIII collagen is a primary regulator of SMC migration and invasion from the media to the intima.

Many matrix molecules that promote cell migration act as attachment factors, because it is necessary for a cell to adhere to a substrate to gain traction for migration.^{37,38} We found dose-dependent increases in both medial and intimal SMC attachment to increasing concentrations of type VIII collagen. However, in general, SMC attachment to type VIII collagen was less than attachment to type I collagen, even when substrate concentrations were normalized on a mole-to-mole basis. This suggests that type VIII collagen was a less adhesive substrate than type I collagen. Because type I collagen is very abundant in the vessel wall, one might predict that very high concentrations of type VIII collagen would have to be present to compete for cell binding. Although we do not know the exact concentration *in vivo*, morphological evidence does suggest that type VIII collagen is deposited in local areas of the vessel after injury. Sibinga et al, using immunohistochemical staining, showed copious deposition of type VIII collagen restricted to medial SMCs immediately subjacent to the lumen and to the developing neointima in the injured rat carotid.¹³ In addition, our results suggest that, at some concentrations, SMCs of the activated intimal

phenotype adhere equally well to type VIII and type I collagens. Thus it is likely that local deposition of high concentrations of type VIII collagen and the presence of activated intimal SMCs combine to make a significant contribution to vascular pathogenesis *in vivo*.

Type VIII collagen acted directly as a chemotactic factor for both medial and intimal SMCs in transwell migration assays. Our checkerboard assay results indicated a true chemotactic effect and not merely a chemokinetic effect, because SMCs migrated along a gradient of type VIII collagen and migration was dramatically diminished when the gradient was abolished. Chemotaxis is an important mechanism stimulating cell migration, and many matrix molecules deposited after injury exert chemotactic effects.^{1,3-5,39-42} Our results can be distinguished from the results of Sibinga et al, in which rat aortic SMCs were shown to migrate across type VIII collagen-coated filters in response to the chemoattractant platelet-derived growth factor-BB suspended in the bottom of the chamber.¹³ We have demonstrated for the first time that type VIII collagen can stimulate chemotaxis directly and independently of other chemoattractant agents.

An early step of SMC migration in the vessel wall is degradation of the matrix by MMPs, which enables cells to break contacts and clears a path for SMC movement from the media into the intima.^{16-18,43,44} In this report, we have shown that type VIII collagen can stimulate MMP-2 and MMP-9 synthesis in intimal SMCs grown in tissue culture. In two very exciting recent studies, Plenz et al reported coexpression of type VIII collagen and MMP-1 by SMCs in the intima of balloon-injured iliac arteries in cholesterol-fed rabbits and by macrophages in human atherosclerotic lesions.^{14,20} These results are consistent with our work, and we have significantly extended this by demonstrating for the first time an important functional role for type VIII collagen in stimulating MMP synthesis in intimal SMCs.

To the best of our knowledge, this is the first report that a matrix molecule can directly stimulate MMP synthesis by SMCs. Furthermore, we found that type I collagen, which is a normal matrix component found in abundance in the uninjured vessel wall, does not stimulate MMP synthesis. We suggest that this feedback regulation of matrix degradation is a property unique to matrix molecules that are induced after injury. We have evidence that osteopontin, another injury-induced matrix molecule, stimulates MMP-1 synthesis (unpublished observation). Evidence for this matrix feedback paradigm is accumulating from studies of several other cell types. Pioneering studies in fibroblasts,²¹⁻²⁴ keratinocytes,^{25,26} melanoma cells,²⁷ macrophages,²⁸ and osteogenic cell lines²⁹ have demonstrated MMP production regulated by feedback from the extracellular matrix.

In the injured vessel wall, the up-regulation of MMP synthesis precedes the appearance of SMCs in the intima, suggesting that medial SMCs are triggered to produce MMPs, and this may be part of the phenotypic shift from a quiescent to active state. Indeed, several growth factors and cytokines that mediate the phenotypic shift are also capable of stimulating MMPs.⁴⁵ We show here

for the first time that type VIII collagen can stimulate MMP synthesis in SMCs that have adopted the activated intimal phenotype. *In vitro*, type VIII collagen did not stimulate MMP synthesis in SMCs derived from a quiescent media, despite similar levels of attachment to substrate and the fact that attachment was mediated by same $\beta 1$ integrin receptor as intimal cells, and this further underlines the possibility of distinct signaling mechanisms in the two phenotypes.

SMCs plated on type VIII collagen formed focal adhesion plaques during cell spreading, suggesting the involvement of integrin receptors in binding. Using three different types of SMCs and integrin blocking antibodies, we determined that adhesion to type VIII collagen was mediated via the $\beta 1$ integrin subunit in rat intimal and medial SMCs and human newborn aortic SMCs. Because antibodies against rat α integrin subunits were not available, we used the human SMCs to determine which α integrins were paired with the $\beta 1$ integrin in binding to type VIII collagen. The $\alpha 2$ integrin was the predominant receptor mediating over 70% of attachment, whereas $\alpha 1$ mediated 30% of the attachment to type VIII collagen. These *in vitro* results should be interpreted with some caution, because a previous report has suggested that SMCs *in vivo* in the rat carotid do not express the $\alpha 2\beta 1$ integrin receptor.⁶ Further work is necessary to determine whether this holds true for all species and tissues.

Our hypothesis is that type VIII collagen functions as a provisional matrix substrate in the vessel wall after injury. During wound healing in the skin, provisional matrices are transiently synthesized and enable attachment, activation, and migration of stromal and inflammatory cells.²⁶ Once tissue healing is complete, these matrices are cleared and replaced by a more permanent matrix. Type VIII collagen is ideally suited to fulfill the role of a provisional substrate, because, in the injured rat carotid, we saw an extraordinarily rapid induction (within 24 hours) and clearance of type VIII collagen from the vessel wall by 7 days after injury.¹² We hypothesize that SMCs adjacent to the vessel lumen and in the neointima increase type VIII collagen synthesis in response to growth factors released at the denuded arterial surface, creating a deposit of provisional matrix that stimulates nearby SMCs and attracts cells from deeper in the media.¹² This is consistent with the pattern of immunohistochemical staining for type VIII collagen in the rat carotid, which was reported by Sibinga et al.¹³ This deposit of activating matrix may then promote cell attachment and stimulate proteinase secretion, facilitating migration into the intima. The protein is easily digested within its globular domains by a variety of serine proteinases and in the collagenous domain by MMPs.⁴⁶ Up-regulation of plasmin and MMPs occurs between 1 and 7 days after arterial injury,^{16,18,47,48} and may provide the mechanism for rapid clearance of type VIII collagen from the vessel.

In conclusion, we report that type VIII collagen is able to support attachment, focal adhesion formation, and spreading of vascular SMCs and acts as a strong chemotactic factor. Furthermore, type VIII collagen stimulates MMP production, suggesting regulation of SMC invasion. These studies suggest that type VIII collagen

plays a critical role in regulating SMC invasion and migration after vascular injury.

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