Migration and Growth Are Attenuated in Vascular Smooth Muscle Cells With Type VIII Collagen-Null Alleles

Eser Adiguzel, Guangpei Hou, Diane Mulholland, Ulrike Hopfer, Naomi Fukai, Bjorn Olsen, Michelle Bendeck

Objective—Type VIII collagen is upregulated after vascular injury and in atherosclerosis. However, the role of type VIII collagen endogenously expressed by smooth muscle cells (SMCs) and in the context of the vascular matrix microenvironment, which is rich in type I collagen, is not known. To address this, we have compared aortic SMCs from wild-type (WT) mice to SMCs from type VIII collagen-deficient (KO) mice when plated on type I collagen.

Methods and Results—Type VIII collagen was upregulated after wounding of WT SMCs. KO SMCs exhibited greater adhesion to type I collagen than WT SMCs (optical density [OD\textsubscript{595}]=0.458±0.044 versus 0.193±0.071). By contrast, the WT SMCs spread more (389±75% versus 108±14% increase in cell area), migrated further (total distance 80.6±6.2 μm versus 64.2±4.4 μm), and exhibited increased [\textsuperscript{3}H]-thymidine uptake (160 000±22 300 versus 63 100±12 100 counts per minute) when compared with KO SMCs. Gelatin zymograms showed that WT SMCs expressed latent matrix metalloproteinase 2, whereas KO SMCs did not. Addition of exogenous type VIII collagen returned levels of KO SMC adhesion (OD\textsubscript{595}=0.316±0.038), migration (79.5±5.8 μm), and latent matrix metalloproteinase 2 expression to levels comparable to WT SMCs.

Conclusions—This study suggests that SMCs can modify the matrix microenvironment by producing type VIII collagen, using it to overlay type I collagen, and generating a substrate favorable for migration. (Arterioscler Thromb Vasc Biol. 2006;26:56-61.)

Key Words: atherosclerosis ■ restenosis ■ collagen ■ smooth muscle cell migration ■ MMP

Collagens compose a large portion of the extracellular matrix in the vessel wall and play important roles maintaining the strength and structural integrity of blood vessels. During atherosclerosis, collagen accumulation contributes to lesion growth and vessel contraction, and collagen degradation leads to plaque instability and rupture.\(^1\) In addition to their roles providing structural support in the arterial wall, in vitro studies suggest that collagens can act as signaling molecules stimulating changes in the phenotype and behavior of smooth muscle cells (SMCs), endothelial cells, and macrophages. Recent research suggests that SMCs respond differently to different types or physical states of collagen. For example, intact type I collagen maintains SMC quiescence, whereas denatured or degraded collagen stimulates proliferation and migration.\(^2-4\)

Type VIII collagen is a member of the short chain collagen family, composed of α1 (VIII) and α2 (VIII) chains. It is produced by corneal and aortic endothelial cells, as well as mesangial cells, and is upregulated during the proliferation of these cells and during angiogenesis.\(^5-8\) In normal arteries, it has been localized to the subendothelial intima and is present in very low amounts in the media and adventitia.\(^9\) Type VIII collagen expression is dramatically increased after balloon injury of the rat carotid\(^10,11\) or the porcine coronary artery.\(^12\) In the rat balloon injury model, type VIII collagen was expressed by SMCs migrating and proliferating during intimal thickening.\(^10,11\) It is also present in the atherosclerotic lesions of apolipoprotein E–deficient mice\(^13\) and cholesterol-fed rabbits subject to balloon injury.\(^14\) Where expression is localized to intimal SMCs and to macrophage-rich areas of the plaque, suggesting that macrophages also produce type VIII collagen. Type VIII collagen was similarly localized in human atherosclerotic plaques.\(^15-17\) Expression of mRNA for type VIII collagen is regulated by platelet-derived growth factor, fibroblast growth factor 2, and angiotensin II, all important factors in the pathogenesis of atherosclerosis.\(^11,18\)

Investigating the interaction of SMCs with exogenous type VIII collagen in vitro, we and others have shown that the protein acts as an attachment and chemotactic factor for SMCs.\(^11,18\) SMCs attach to type VIII collagen, but it is a less adhesive substrate and promotes greater cell migration than type I collagen. In addition, type VIII collagen stimulates...
SMC matrix metalloproteinase synthesis, whereas type I collagen does not. These studies were performed using exogenous type VIII collagen coated on tissue culture plates as a substrate for the SMCs. However, in the diseased vessel wall, type VIII collagen is expressed and deposited by SMCs in the presence of an existing matrix rich in type I collagen. The function of endogenously expressed type VIII collagen in this more complex matrix microenvironment has not been studied. We now hypothesize that after arterial injury, SMCs produce type VIII collagen and use it to overlay an existing extracellular matrix, providing a substrate more favorable for rapid migration. To address this hypothesis, we have compared aortic SMCs isolated from Col8a1+/−/Col8a2+/− mice [wild-type (WT)] to SMCs isolated from type VIII collagen–deficient mice, Col8a1−/−/Col8a2−/− (KO), to examine different components of the migratory process when the cells are plated on either uncoated or type I collagen–coated surfaces.

Methods

For a detailed account of the methodologies used in this article, please see http://atvb.ahajournals.org.

Mice with targeted deletion of both the Col8a1 and Col8a2 genes (KO) were generated in the laboratory of Bjorn Olsen (Harvard Medical School) as described with WT littermate mice used as controls. Aortic vascular SMCs were isolated from the mice as described previously. For all of the experiments, tissue culture flasks/plates were either left uncoated or coated with a solution containing 50 μg/mL of pepsin-solubilized bovine dermal type I collagen (Vitrogen 100; Collagen Biomaterials). Cell attachment assays were performed as described previously. For spreading assays, 100,000 cells were seeded onto 25-cm² tissue culture flasks and imaged using a Nikon Eclipse TE2000 inverted microscope equipped with a heated stage. A Hamamatsu digital camera (model #C4742-95) was used to capture images every 10 minutes for 4 hours after plating. Three to 6 cells were analyzed for each experiment. Migration assays were similar, with the following modifications: 100,000 cells/well were seeded onto 6-well plates, then grown until 50% confluence (0.5 to 2 days), and, subsequently, images of migrating cells were captured every 10 minutes for 8 hours. Six to 8 cells were analyzed in each experiment. SMC proliferation was estimated using [3H]-thymidine incorporation. MMP activity in the SMC-conditioned medium was assayed using gelatin zymograms as described previously. Rescue experiments using KO SMCs with the addition of exogenous type VIII collagen were also performed. Wells were first coated with a solution containing 37.5 μg/mL type I collagen/PBS then rinsed with PBS and coated with 6.6 μg/mL exogenous type VIII collagen/PBS (type VIII collagen was isolated from bovine Descemet’s membrane as described previously). This gives a coating composed of 75% type I collagen and 25% type VIII collagen, with the same total molar concentration as the 50 μg/mL type I collagen used in the first experiments. Adhesion and migration experiments were performed on this mixed collagen substrate as described above. Immunocytochemistry was done using an antigelatin α/1 (VIII) monoclonal antibody (Clone 8C, Seikagaku America).

Results

SMC Morphology Differed Between KO and WT SMCs

Western blots probed with an antibody against type VIII collagen revealed a band of 240 kDa in the lysates from WT SMCs, whereas this band was absent in KO SMCs (Figure IA, available online at http://atvb.ahajournals.org). SMCs obtained from WT and type VIII collagen KO mice exhibited distinct morphologies in culture. When plated on uncoated wells, WT SMCs appeared small and elongated, usually displaying only 1 or 2 long cytoplasmic protrusions (Figure IB). By contrast, KO SMCs were larger and rounder, and they extended thin, short, stellate processes (Figure IC). Morphometric measurements demonstrated that KO SMCs were indeed significantly larger (2830±439 μm² versus 649±61 μm²; P ≤ 0.001) and rounder (0.153±0.021 versus 0.112±0.008; P = 0.039, with a value of 1 corresponding to a perfect circle) than WT SMCs. KO SMCs plated on polymerized type I collagen exhibited a similar morphology to those plated on plastic, whereas WT SMCs plated on type I collagen were more rounded with no protrusions visible (data not shown). There was no difference in viability between KO and WT SMCs, whether they were plated on uncoated or on type I collagen-coated wells (data not shown).

Figure 1. Immunostaining revealed type VIII collagen in WT SMCs as punctate cytoplasmic immunostaining on injury (A) that was upregulated 24 hours after injury (C). No staining was evident in KO SMCs on injury (B) or 24-hours later (D). W, wounded area; scale bar, 100 μm.

Production of Type VIII Collagen Was Upregulated After Injury

Confluent layers of SMCs were subject to a scrape wound, then immunostained with an antibody against type VIII collagen. Immediately after wounding, WT SMCs in the uninjured monolayer and in areas adjacent to the wound stained for type VIII collagen (Figure 1A), whereas KO SMCs did not stain (Figure 1B). Type VIII collagen was localized in the cytoplasm with a punctate staining pattern. Double staining with an antibody raised against a marker of the Golgi complex (58K Golgi protein marker) revealed that most of the intracellular type VIII collagen was localized in the Golgi (data not shown). A substantial increase in type VIII collagen was evident in the WT cells 24 hours after wounding (Figure 1C). By contrast, KO SMCs did not stain for type VIII collagen at 24 hours (Figure 1D).

We had some difficulty staining type VIII collagen within the extracellular matrix, probably because the protein is tightly complexed with other matrix proteins. However, after lightly digesting the matrix with 10 μg/mL pepsin in 0.1 mol/L acetic acid, we were able to detect extracellular
immunostaining for type VIII collagen (Figure 2A). Furthermore, after treating confluent cultures with a mixture of EDTA/EGTA to lift off cells, then lysing the underlying matrix, we were able to detect matrix-bound type VIII collagen produced by WT cells but not KO cells on a Western blot (Figure 2B).

Production of Type VIII Collagen Decreased Attachment of SMCs to Type I Collagen and Facilitated Spreading and Migration

SMCs must attach to substrate to gain traction for migration; however, too strong an attachment may fix the cells in place and prevent migration. To determine the effect of type VIII collagen production on SMC adhesion, we measured adhesion to uncoated wells or to wells coated with type I collagen. The WT SMCs adhered significantly less than KO SMCs to uncoated wells (Figure 3A) or to wells coated with type I collagen (Figure 3B). The difference in adhesion was especially large when the cells were plated on type I collagen (optical density [OD]$_{595}$=0.193±0.071 for WT cells versus OD$_{595}$=0.458±0.044 for KO cells). The addition of exogenous type VIII collagen to the wells to rescue the phenotype resulted in the decreased attachment of KO cells (OD$_{595}$=0.316±0.038) to a level that was not significantly different from WT cells (Figure 3B).

Cell spreading and protrusion occur during migration and are also affected by adhesion strength, so we compared the spreading in WT and KO SMCs. Our preliminary experiments revealed that most spreading occurred during the first hour after plating. When plated on uncoated flasks, WT SMCs spread and increased cell area by ~4-fold in 1 hour (370±61%), significantly more than KO SMCs, which increased in area by only 2-fold (206±37%; Figure 4A). WT SMCs also spread ~4-fold in 1 hour when plated on 50 μg/mL type I collagen (389±75%), significantly more than KO SMCs, which did not spread on type I collagen (108±14%; Figure 4B).

We used time-lapse microscopy to measure cell migration. The total distance migrated by individual cells over an 8-hour period was calculated. Whether plated on uncoated or type I collagen–coated wells, the distance traveled by WT SMCs was significantly greater than that for KO SMCs (Figure 5). When plated on uncoated wells, WT SMCs traveled a total distance of 115±9 μm compared with 72.7±3.8 μm for KO SMCs (Figure 5A). When plated on 50 μg/mL type I collagen, WT SMCs traveled a total distance of 80.6±6.2 μm compared with 64.2±4.4 μm for KO SMCs (Figure 5B). The addition of type VIII collagen to the plates rescued the KO SMC migration such that the KO rescue (KO-R) SMCs traveled a distance not different from the distance traveled by WT SMCs (79.5±5.8 μm; Figure 5B).

Type VIII Collagen Production Increases MMP Activity

Because MMPs facilitate migration of SMCs by allowing the clearance of matrix barriers, gelatin zymograms were used to measure MMP-2 and MMP-9 activity in conditioned media from WT and KO SMCs (Figure 6A). Conditioned media from mouse embryonic fibroblasts (MEFs) was used as a positive control and to identify the lytic bands on zymogram gels. MEF-conditioned media contained distinct lytic bands at 95 kDa (active MMP-9), 70 kDa (latent MMP-2), and 61 kDa (active MMP-2). Media from WT and KO mouse SMCs contained lytic bands of 106 kDa (latent MMP-9), 95 kDa (active MMP-9), 84 kDa (unknown), 77 kDa (unknown), and 70 kDa (latent MMP-2). There was increased lysis in the latent MMP-2 band in the conditioned media of WT SMCs compared with the conditioned media of KO SMCs. The addition of exogenous type VIII collagen to the plate led to an increase in latent MMP-2 production by KO cells, showing a complete rescue of the KO phenotype. By contrast, there were no apparent differences in the activity of MMP-9 or the unidentified bands, comparing WT and KO SMCs. Within each cell type, there were no differences in MMP activity between cells plated on plastic or on type I collagen (data not shown).

Type VIII Collagen Facilitates SMC Proliferation

To assess cell proliferation, [3H]-thymidine incorporation was measured. Thymidine incorporation was similar in WT and KO SMCs plated on plastic (Figure 6B). By contrast, thymidine incorporation was significantly higher in WT than KO SMCs plated on type I collagen (95.0±7.8 cpm for WT versus 48.2±3.8 cpm for KO; Figure 6C).
dine uptake was increased in WT SMCs plated on type I collagen (160,000±22,300 counts per minute) compared with KO SMCs (63,100±12,100 counts per minute).

**Discussion**

Knowledge about the function of type VIII collagen is scarce. However, it is expressed at high levels in injured arteries and in the atherosclerotic lesions of humans. Previous studies using differential display PCR described the upregulation of type VIII collagen in injured compared with uninjured rat carotid arteries. Type VIII collagen was deposited in copious amounts by SMCs immediately subjacent to the vessel lumen and in SMCs forming thickened neointimal lesions after injury, a pattern that correlated with SMC migration. Previously, we performed in vitro experiments to study the interactions of SMC with exogenous type VIII collagen and demonstrated that the protein was an adhesive and chemotactic substrate and that it also stimulated MMP synthesis by neointimal SMCs. Taken together, these data suggested an important role for type VIII collagen in promoting SMC migration.

However, the vascular extracellular matrix is a complex mixture composed of several different types of molecules, and it is particularly rich in type I collagen. In fact, type I collagen and type VIII collagen are both upregulated and colocalized during plaque development. In vitro studies have shown that SMCs can attach to type I collagen; nonetheless, a substantial body of evidence shows that intact polymerized type I collagen inhibits cell migration and proliferation and downregulates the expression of many genes. By contrast, type VIII collagen appears to stimulate opposite responses. Although the effects of exogenous type VIII collagen on SMCs have been studied, the importance of endogenously produced type VIII collagen is not known. Furthermore, the effect of type VIII collagen in the presence of a polymerized type I collagen matrix has not been examined. In the current study, we hypothesized that SMCs produce type VIII collagen, lay it down on top of type I collagen, and use this modified, less adhesive matrix to facilitate migration. To investigate this, we studied SMCs harvested from the aortas of WT and type VIII collagen KO mice. We compared the ability of these cells to migrate on dishes coated with polymerized type I collagen, which was used to mimic the natural environment encountered in the vascular media.

KO SMCs displayed significantly stronger attachment than WT SMCs to both tissue culture plastic and to wells coated with type I collagen substrate. This suggests that cells that are able to produce type VIII collagen adhere less, and these results are in accordance with our previous studies where we found that SMC attachment to type VIII collagen was less than attachment to type I collagen. In fact, when KO SMCs were plated on a mixture of type VIII and type I collagen, their levels of attachment were reduced to a level comparable to WT SMCs, showing a partial rescue of the KO phenotype.

To accomplish migration in a defined direction, cells first extend leading lamellopodia, which attach to the substrate. After this is cell contraction, then attachments to the substrate
SMCs when plated on type I collagen. This suggests that endogenously produced type VIII collagen allows cells to overcome the inhibitory effects of type I collagen on proliferation. Likewise, type VIII collagen has recently been implicated in stimulation of the proliferation of corneal endothelial cells. The production of matrix-degrading enzymes, such as the MMPs, is required for SMCs to detach from matrix to migrate or proliferate and to facilitate the clearance of matrix barriers. Gelatin zymograms revealed MMP-2 activity in the media from WT SMCs, whereas there was less MMP-2 activity in the media from the KO SMCs. However, the addition of exogenous type VIII collagen to the KO SMCs increased the MMP-2 production by these cells. These results confirm previous studies where we showed that type VIII collagen stimulated the production of both MMP-2 and MMP-9 by rat SMCs. However, we did not see a difference in MMP-9 activity in the mouse cells, suggesting that there may be species-specific differences.

Type VIII collagen interacts with SMCs via the αβ integrins and the recently discovered discoidin domain receptor tyrosine kinases (DDR1 and 2). Both integrins and DDR1 control cell proliferation on collagenous substrates and can activate intracellular signaling pathways leading to the upregulation of MMPs. Furthermore, both DDR1 and the αβ integrin are upregulated after arterial injury in coincidence with type VIII collagen.

Our studies have concentrated on the SMC as the source of type VIII collagen and focused on SMC interactions with this protein. However, other cell types in the vessel wall produce type VIII collagen in the atherosclerotic plaque, including endothelial cells and macrophages. At this time, very little is known about the interactions of these cells with the protein, but these interactions are also likely to be important in mediating the injury response in vascular disease.

The results presented here show that vascular SMCs derived from mice with targeted deletion of type VIII collagen exhibit critical defects in migration and proliferation. Furthermore, the ability of these cells to express MMP-2 is reduced compared with WT SMCs, which produce type VIII collagen. This reduction in MMP-2 activity and migration over a type I collagen matrix was reversed with the addition of exogenous type VIII collagen. These studies suggest that SMCs are able to produce type VIII collagen and use it to overlay type I collagen, providing a provisional substrate favorable for migration. Thus, type VIII collagen may be an important mediator of SMC responses in vascular diseases that involve cell migration, including atherosclerosis, restenosis, vein graft, and transplant atherosclerosis.

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References


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FIGURE I

A. A 240 kDa band representing type VIII collagen was present within cell lysates from WT SMCs, but absent in cell lysates from KO SMCs. Differences in cell morphology were evident between cultures of WT SMCs (B), and KO SMCs (C). Scalebar represents 100 mm.
ONLINE METHODS

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO), except where noted otherwise.

Mice and cell culture

Mice with targeted deletion of both the *Col8a1* and *Col8a2* genes (KO) were generated in the laboratory of Dr. Bjorn Olsen (Harvard Medical School) as described\(^1\) with wild-type littermate mice (WT) used as controls. Genotypes were verified using extracted tail DNA and polymerase chain reaction (PCR) for both the *Col8a1* and *Col8a2* alleles. The primers for wild-type *Col8a1* were as follows: sense, 5’-CGG GAG TAG GAA AAC CAG GAG TGA-3’; antisense, 5’-GGC CCA AGA ACC CCA GGA ACA-3’. Total length of product is 313 bp. The primers for the knockout *Col8a1* were as follows: sense, 5’-GTG GGG GTG GGG TGG GAT TAG ATA-3’; antisense, 5’-CTC GGC CCA AGA ACC CCA GGA AC-3’. Total length of product is 503 bp. The primers for wild-type *Col8a2* were as follows: sense, 5’-CCG GTA AAG TAT GTG CAG C-3’; antisense, 5’-CAA GTC CAT TGG CAG CAT C-3’. Total length of product is 690 bp. The primers for knockout *Col8a2* were as follows: sense, 5’-CAG CGC ATC GCC TTC TAT CGC-3’; antisense, same as the wildtype *Col8a2* antisense primer. Total length of product is 1200 bp.

Aortic vascular SMCs were isolated from the mice as previously described.\(^2\) Cells were maintained in 10% fetal calf serum and 2% penicillin-streptomycin supplemented Dulbecco’s Modified Eagle’s Medium (DMEM), (10% FCS-DMEM) and used between passage 5-10 for experiments. Unless otherwise noted, all experiments were performed with cells in 10% FCS-DMEM. For all experiments, tissue culture plates/flasks were either left uncoated, or coated...
with a solution containing 50 µg/mL of pepsin-solubilized bovine dermal type I collagen (Vitrogen 100; Collagen Biomaterials, Mahwah, NJ). Vitrogen 100 stock solution was dissolved in phosphate-buffered saline (PBS) and neutralized with NaOH. Unless otherwise described, plates/flasks were then incubated for 1 hour at 37°C and then blocked with 10 mg/mL bovine serum albumin (BSA)/PBS.

Cell morphology

5,000 SMCs/well were plated in 6 well plates (uncoated) and allowed to attach for 16 hours. Cells were then fixed and stained with the Dif-Quik Stain Set (Dade Behring, Newark, DE). Cells were imaged using a Nikon Eclipse TE200 inverted microscope, Hamamatsu camera (model # C4742-95), and Simple PCI software. Simple PCI software was used to measure cell area by tracing around the outside edge of the cell, and calculating the area within. Roundness was calculated using the formula:

\[
\text{Roundness} = \frac{4\pi \text{Area}}{\sqrt{\text{perimeter}}}
\]

Immunocytochemistry

12 mm round glass coverslips (Fisher Scientific) were placed in 24-well plates and 50,000 cells/well were seeded and grown to confluence. A scrape-wound was created in the monolayer by dragging a 200 µL micropipette tip across the coverslip. The cells were washed twice with HBSS, and 1% FCS-DMEM was added. At 0 or 24 hours after wounding, cells were rinsed twice with PBS, then fixed with 4% paraformaldehyde. SMCs were stained with anti-collagen α1(VIII) (Clone 8C, Seikagaku America, East Falmouth, MA)mAb at a dilution of 1:500 using a monoclonal antibody detection kit with AEC Chromagen (R&D Systems,
Minneapolis, MN). SMCs were then counterstained with hematoxylin Quick Stain (Vector, Burlington, Ontario), and mounted on slides under 1:1 PBS:glycerol.

To localize intracellular type VIII collagen, cells fixed as above were double-stained with anti-58K Golgi protein (Abcam, Cambridge, MA) and a Cy3-conjugated anti-rabbit secondary (Jackson Immunologicals, West Grove, PA), and anti-collagen α1(VIII) and an FITC-conjugated anti-mouse secondary antibody, all at a dilution of 1:250, and mounted on slides under Prolong Antifade Gold mounting medium (Molecular Probes, Eugene, OR).

To localize extracellular type VIII collagen, 22 mm square glass coverslips were placed in 6-well plates and 3,000 cells/well were seeded, and grown to confluence for 21 days. The cells were rinsed twice with PBS, and incubated with 10µg/ml pepsin in 0.1 M acetic acid for 5 minutes at 37°C. The matrix was fixed with 4% paraformaldehyde, then stained with the type VIII collagen antibody and an FITC-conjugated anti-mouse secondary antibody, and mounted on slides under Prolong Antifade Gold. Slides were imaged with a Nikon Eclipse E600 microscope, Hamamatsu camera and Simple PCI software (Compix Inc., Mars, PA).

Adhesion assays

96-well plates were either uncoated or coated with type I collagen, then incubated at 4°C for 16 hours. 60,000 cells/well for uncoated wells and 30,000 cells/well for type I collagen-coated wells were seeded and incubated for 1 hour at 37°C and adhesion assays were performed as previously described.3 Briefly, 60,000 cells per well for uncoated wells, and 30,000 cells per well for type I collagen-coated wells were allowed to adhere for 1 hour at 37°C, after which non-adherent cells were washed off with PBS. Adherent cells were fixed and stained with 0.5% toluidine blue dissolved in 4% paraformaldehyde, then solubilized with 1% sodium dodecyl
sulphate (SDS), and absorbance was read on a spectrophotometer (Molecular Devices, Sunnyvale, CA) at 595 nm.

*Spreading and migration assays*

For spreading assays, 100,000 cells/flask were seeded onto 25 cm² tissue culture flasks and imaged using a Nikon Eclipse TE200 inverted microscope equipped with a heated stage. A Hamamatsu digital camera was used to capture images every 10 minutes for 4 hours after plating. 3-6 cells were analyzed for each experiment. Migration assays were performed similar to the spreading assays, with the following modifications: 100,000 cells were seeded onto 6-well plates, then grown until 50% confluent (0.5-2 days). Subsequently, images were captured every 10 minutes for 8 hours. 6-8 cells were analyzed in each experiment. Spreading was expressed as the percent change in cell area relative to start, and migration was measured as the total distance traveled over time, using Simple PCI software.

*Gelatin zymography*

50,000 SMCs/well were plated onto 6 well plates, and allowed to attach for 16 hours. Wells were then washed with Hank’s Balanced Salt Solution (HBSS), followed by incubation for 24 hours with 1 mL of serum-free DMEM containing 2% BSA. The conditioned media was collected, and used for MMP analysis by gelatin zymography. Conditioned media from mouse embryonic fibroblasts (MEFs) stimulated with cytochalasin D was used as a positive control (provided by Dr. Rama Khokha, University of Toronto). 10 µL of conditioned media was loaded into separate wells on an 8% SDS-polyacrylamide gel containing 0.1% gelatin as a substrate for MMP activity and zymograms were processed as previously described.⁴
**Rescue experiments**

We attempted to rescue the KO SMCs by adding exogenous type VIII collagen (isolated from bovine Descemet’s membrane as previously described\(^5\)) to the type I collagen coated plates. Wells were first coated with a solution containing 37.5 µg/mL type I collagen/PBS, and incubated at 37º C for 1 hour. The wells were then rinsed with PBS and coated with 6.6 µg/mL exogenous type VIII collagen/PBS. This gives a coating composed of 75% type I collagen and 25% type VIII collagen, with the same total molar concentration as the 50 µg/mL type I collagen used in the first experiments. Adhesion, migration, and gelatin zymography were performed on this mixed collagen substrate as described above.

**Proliferation assay**

To measure proliferation, SMCs were plated at a density of 10,000 cells per well and allowed to attach for 48 hours. To growth arrest and synchronize the cells, the SMCs were then incubated for 24 hours with serum-free DMEM containing 2% BSA, which was followed by replacement of the media with 10% FCS-DMEM supplemented with 2 µCi/mL \(^3\)H-thymidine (Amersham Biosciences, Piscataway, NJ). 48 hours later, wells were washed with PBS, then fixed with 10% trichloroacetic acid (TCA). The wells were then washed with 10% TCA and 95% ethanol. Wells were then incubated with 0.3M NaOH and subsequently neutralized with the addition of 0.3N HCl. The contents of the well were transferred to scintillation vials and Cytoscint (ICN Biomedical, Irvine, CA) was added. Counts per minute (CPM) were measured using a liquid scintillation counter (Fisher Scientific, Markham, ON).
Western blotting

Confluent cultures of SMCs grown in 75 cm² flasks with 10%-FCS-DMEM were lysed with 50 mM Tris (pH 7.6), 0.1% SDS, 0.1 mM PMSF, and 10 µg/mL leupeptin. Total protein levels in the cell lysates were determined using a protein assay kit (DC Protein Assay, BioRad, Mississauga, ON) and 10 µg of protein was subjected to 8%-acrylamide SDS-PAGE. Western blots were probed with anti-collagen α1(VIII) mAb diluted 1:500. Western blots were developed using horseradish peroxidase–coupled secondary sheep anti-mouse Ig Ab and enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA). Some cultures were grown for 21 days, when they were treated with a mixture of 10mM EDTA and 10mM EGTA in PBS to lift cells off the matrix, the matrix was then scraped off the plate in lysis buffer, and 10 µg of matrix protein was subjected to gel electrophoresis and western blotting for type VIII collagen as described above.

Cell viability assays

Cell viability assays were performed using 96-well tissue culture plates. Cells were plated at densities of 500, 1,000, 2,500, 5,000, 7,500, or 10,000 cells per well and incubated at 37°C for 24 hours. Cell viability assays were performed using a Colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) Assay for Cell Survival (Chemicon International Inc, Temecula, CA) with four hours of incubation between the addition of Solutions AB and C. Plates were read on a spectrophotometer at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Statistics
Each experiment was repeated in duplicate or triplicate. The cell morphology data were analyzed by Student’s T-test. Other data were analyzed by ANOVA, with the exception of the migration data, which were analyzed with repeated measures ANOVA. Student-Newman-Keuls post-hoc tests were used to determine statistical significant differences between groups (SigmaStat v3.1, Systat Software Inc., Point Richmond, CA).

Reference List


