A Quantitative In Vitro Model of Smooth Muscle Cell Migration Through the Arterial Wall Using the Human Amniotic Membrane

Klaus Kallenbach, Harold A. Fernandez, Graziano Seghezzi, F. Gregory Baumann, Sundeep Patel, Eugene A. Grossi, Aubrey C. Galloway, Paolo Mignatti

Objective—The development of intimal hyperplasia involves smooth muscle cell (SMC) migration into the intima and proliferation. Matrix metalloproteinases and their tissue inhibitors play important roles in this process. In this study, we describe a novel in vitro model for studying SMC migration through the vessel wall.

Methods and Results—Human aortic SMCs (hASMCs) labeled with 125I-iododeoxyuridine or unlabeled were grown on the stromal aspect of the human amniotic membrane. Mechanical damage to endothelial cells grown on the basement membrane and addition of growth factors or platelets were characterized for their effect on SMC migration into the stroma both by histological methods and by measuring the radioactivity associated with the membrane after removal of noninvasive SMCs. To assess the reliability of the model, the cells were infected with a recombinant adenovirus encoding the tissue inhibitor of metalloproteinase-1 (TIMP-1). Addition of a platelet-derived growth factor gradient stimulated hASMC infiltration into the stroma. This effect was abolished with TIMP-1–transduced hASMC, confirming that TIMP-1 overexpression blocks SMC invasion of the stroma.

Conclusions—This in vitro model of SMC migration in the vessel wall provides an inexpensive, quantitative, and reliable tool to study the molecular and cellular mechanisms of intimal hyperplasia. (Arterioscler Thromb Vasc Biol. 2003;23:1008-1013.)

Key Words: smooth muscle cells --- migration --- intimal hyperplasia --- tissue inhibitor of metalloproteinase-1 --- human amnion

Smooth muscle cell (SMC) proliferation and migration from the tunica media to the intima are key mechanisms of intimal hyperplasia and atherosclerosis. These cell functions are controlled by several growth factors and require the action of extracellular matrix (ECM)-degrading proteinases. Platelet-derived growth factor (PDGF) and basic fibroblast growth factor (FGF-2) stimulate SMC migration and proliferation in tissue culture and in injured vessels. However, growth factors alone do not stimulate SMC migration or proliferation in organ cultures or uninjured vessels. A variety of extracellular proteinases are involved in vascular remodeling. Among them, the matrix metalloproteinases (MMPs) have important roles. In extracellular spaces, MMP activity is controlled by tissue inhibitors of metalloproteinases (TIMPs), which are secreted by a variety of cells, including SMC. Retrovirus-mediated overexpression of TIMP-1 in SMC reduces SMC invasion of ECM in vitro and migration into the intima in vivo.

A variety of experimental models has been developed to study neointima formation in vivo or in vitro. Animal models afford reproducing human lesions but have major disadvantages, including high cost, the high variability intrinsic with animal models, species variability, and difficulty to quantitate the results in an objective, observer-independent manner. Conversely, in vitro models afford quantitative characterization of select pathogenetic components of intimal hyperplasia, eg, smooth muscle cell proliferation or migration, but are limited by their artificial conditions. The structure and composition of the substrates used for studying ECM degradation or cell migration in vitro (eg, Matrigel) differ considerably from their in vivo counterparts. We attempted to develop a simple and reproducible model for intimal hyperplasia in vitro using a natural ECM. In this study, we describe an in vitro model of the arterial wall using the human amniotic membrane to characterize SMC migration through a natural collagenous stroma. To test the reliability of our model, we characterized the effect of factors that affect formation of intimal hyperplasia in vivo, including the inhibitory effect of TIMP-1 overexpression. The results show that this in vitro set up can reliably be used to reproduce the
of this culture chamber is the stromal aspect of the amnion, the wall is the inner wall of the silicone rubber ring (Figure 1A). Unlabeled or 125I-labeled hASM or BSMC (3×10^5 cells/0.2 mL of medium) were seeded into these culture chambers and allowed to attach to the amnion stroma for 1 hour at 37°C. The silicone rings were removed and placed on the bottom of the plastic wells, and the membrane-ring setups were turned over and placed onto the silicone rings. One milliliter of growth medium containing 3×10^5 BAEC was added onto the basement membrane inside the Teflon rings, and 3 mL of complete medium was added to the outside of the rings so that no hydrostatic pressure would be exerted on the amniotic membrane (Figure 1B). After 2 days of incubation at 37°C, when the endothelial cells formed a confluent monolayer, either 10 ng/mL of PDGF (Sigma Chemical Co) or 2×10^4 human platelets or 10 ng/mL of FGF-2 (kindly provided by Dr D.B. Rifkin, NYU School of Medicine, New York, NY) or an equivalent volume of control medium was added into the upper compartment of the culture chambers. In some experiments, the endothelial cells were omitted, and 1 mL of medium without cells was added onto the amnion basement membrane. After the indicated incubation times, the cultures were fixed in 3% phosphate-buffered glutaraldehyde for histological examination. When 125I-labeled SMCs were used, the cells were labeled with 125I-dUR and the amnion cultures were processed as described19,20 (please see the online supplement, available at http://atvb.ahajournals.org).

Construction of Recombinant Adenoviral Vectors and Characterization of Transduced Cells
Confluent hASMcs were transduced with recombinant adenoviruses encoding TIMP-1 (Ad.TIMP-1) or β-galactosidase (Ad.βgal)21,22 and characterized for TIMP-1 expression by Northern and Western blotting and by reverse gelatin zymography, as described (please see the online supplement).

Statistical Analysis
Statistical analysis was performed by the ANOVA test for comparison of multiple groups. P<0.05 was considered significant.

Results
Characterization of the In Vitro Reconstituted Arterial Wall
The fetal aspect of the human amnion membrane is covered with a monolayered cuboidal epithelium. After lysis with NH4OH and scraping as described in Methods, virtually all epithelial cells were removed and the basement membrane was completely denuded with no microscopically detectable damage (Figures 2A through 2C). The amnion basement membrane, ~10 μm in thickness, is similar in structure and composition to a vascular basal lamina. The constituents of the 500-μm-thick stroma include collagen types I, III, V, VII, and XII, fibronectin, and laminin-1 and -5,18,23-27 Movat staining for elastin showed that the stroma of the amniotic membrane contains a meshwork of elastic fibers (Figures 2A and 2B). The stroma also contains occasional fibroblasts that were not lysed by treatment with NH4OH. However, consistent with previous reports,29 by electron microscopy no signs of viability were observed in these cells (data not shown).

hASMcs seeded onto the stromal aspect of the amnion spread on the substrate within 1 hour and remained attached after the amnion-ring setup was turned over. The cells proliferated and formed a confluent monolayer of noninvasive cells within 72 hours after seeding (Figure 2C). Incubation for as many as 12 days did not affect the integrity of the monolayer, and occasional hASMcs were rarely seen invad-
ing into the stroma. BAECs seeded onto the basement membrane also formed a noninvasive, sealed monolayer (Figure 2D). Similar results were obtained with human aortic endothelial cells (data not shown).

To characterize factors that stimulate SMC invasion into the stroma, we tested several experimental conditions (Table). Human or bovine aortic SMCs grown on the amnion could be stimulated to migrate into the stroma by addition of 10 ng/mL of PDGF in the upper compartment to create a gradient. In contrast, addition of PDGF to the lower compartment did not increase migration. Addition of 10% FBS to the growth medium was also ineffective. To simulate intimal injury, we scratched the endothelial cell monolayer with the blunt edge of a forceps and measured BSMC migration into the amniotic stroma after 6 days by histological methods and by labeling with 125I-dUR.

Table: Factors Controlling SMC Invasion of the Amnion Stroma

<table>
<thead>
<tr>
<th>Experimental Setting</th>
<th>Number of SMCs in the Stroma*</th>
<th>125I cpm, % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control medium</td>
<td>0.5±0.5</td>
<td>12.5±0.4</td>
</tr>
<tr>
<td>PDGF 10 ng/mL</td>
<td>27.0±2.0</td>
<td>32.0±0.3</td>
</tr>
<tr>
<td>Platelets (2×10⁹)†</td>
<td>21.1±3.2</td>
<td>ND</td>
</tr>
<tr>
<td>FBS 10%</td>
<td>0.7±0.6</td>
<td>ND</td>
</tr>
<tr>
<td>FGF-2 10 ng/mL</td>
<td>1.0±0.7</td>
<td>ND</td>
</tr>
<tr>
<td>FGF-2 50 ng/mL</td>
<td>0.8±0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Endothelial injury</td>
<td>0.6±0.3</td>
<td>10.2±0.3</td>
</tr>
<tr>
<td>Endothelial injury+PDGF 10 ng/mL</td>
<td>30.0±0.3</td>
<td>30.1±0.4</td>
</tr>
<tr>
<td>Endothelial injury+platelets (2×10⁹)</td>
<td>27.0±0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

Mean and SD of triplicate samples are shown. The cells were counted after 6 days of incubation.

ND indicates not determined.

*Washed fresh human platelets free of preservatives, used within 2 hours after isolation.

Injury of the endothelium alone did not affect BSMC migration. When the endothelium was injured in the presence of either 10 ng/mL of PDGF or 2×10⁹ platelets, migration was similar to but not significantly higher than in the absence of injury. Regardless of the experimental setting, human or bovine SMCs were unable to migrate through the basement membrane into the endothelial cell monolayer.

These results showed that endothelial damage per se does not stimulate smooth muscle cell migration, FGF-2 released from damaged endothelial cells or added exogenously does not affect SMC migration, and PDGF is a major inducer of SMC migration in the stroma. Because under our experimental conditions SMC migration was not affected by the presence of an endothelial cell layer on the basement membrane, subsequent experiments were done in the absence of endothelial cells.

Effect of TIMP-1 Overexpression on Vascular Remodeling

To characterize the role of TIMP-1 in SMC migration in our model, we transduced hASMCl with Ad.TIMP-1 or, as a control, with Ad.Bgal. By Northern blotting, cells transduced with Ad.TIMP-1 expressed high amounts of recombinant 1.1 kb TIMP-1 mRNA. Western blotting with TIMP-1 antibody and reverse gelatin zymography showed considerably higher levels of TIMP-1 in the conditioned medium of Ad.TIMP-1–transduced hASMCl than in control or Ad.Bgal-infected cells (online Figure I, available at http://atvb.ahajournals.org). Thus, hASMCl transfection with Ad.TIMP-1 resulted in overexpression of functional TIMP-1. The growth rate of virus-transduced cells was slightly but not significantly higher than that of nontransduced cells (data not shown). To characterize the effect of TIMP-1 gene transfer on SMC migration, transduced hASMCl were seeded onto the stromal aspect of the amniotic membrane and migration was measured. TIMP-1–transduced SMCs, as well as nontransduced or control Ad.Bgal-infected cells, formed a confluent monolayer of noninvasive cells within 72 hours after seeding. In the presence of 10 ng/mL PDGF, untransduced or Ad.Bgal-
transduced hASMCs migrated into the collagenous stroma to the same extent. Cells were found at varying depth in the stromal meshwork, with some cells adhering to the basement membrane (Figures 3A and 3C). Conversely, only occasional superficial Ad.TIMP-1–transduced hASMCs were detected in the stroma in the presence of a PDGF gradient (Figure 3B). In 10 random microscopic fields (×200), only single TIMP-1–transduced hASMCs were found in the stroma after 3 or 6 days of incubation. In contrast, up to 20-fold more nontransduced or Ad.βgal-transduced hASMCs were present in the membrane under the same conditions (P<0.001) (Figure 3D). To quantitate SMC migration in an observer-independent manner, 125I-dUR–labeled hASMCs were grown on the amnion stroma for 3 days and the radioactivity associated with the membrane was measured after removal of the noninvasive monolayer as described19,20 (please see the online supplement). As shown in Figure 4, with nontransduced hASMCs in the absence of PDGF, the radioactivity associated with the membrane was 14.2±3.77% of the total radioactivity (mean±SEM). This value increased 2.1-fold to 30.6±3.12% (P<0.05) in the presence of 10 ng/mL PDGF, indicating that a large number of hASMCs migrated into the amnion stroma. With Ad.TIMP-1–transduced cells, the radioactivity associated with the membrane was 6.9±5% of the total radioactivity, ie, significantly lower than with control, nontransduced (P<0.01) or Ad.βgal-transduced hASMCs (25.6±5.6%, P<0.05).

**Discussion**

A variety of in vivo and in vitro models have been developed to study smooth muscle cell migration during the development of intimal hyperplasia. However, all suffer from major disadvantages, including the high cost and variability of...
animal models, difficulty to quantify the results in an objective, observer-independent manner, or the artificial conditions of in vitro models. In an attempt to develop a simple and reproducible model for intimal hyperplasia, we adapted an in vitro assay that had been developed to study tumor invasion and angiogenesis using a natural basement membrane and stroma. Indeed, neointima formation involves tissue remodeling processes that share several features with tumor invasion and angiogenesis. The data reported here show that the human amniotic membrane affords engineering an in vitro model of the vessel wall to study vascular remodeling in vitro. Our in vitro model has several advantages. First, it is inexpensive. One human amniotic membrane provides a large surface of basement membrane and stroma that can be used for assaying as many as 100 samples. Cocultures of endothelial and smooth muscle cells on the amniotic membrane can be maintained for at least 14 days without significant decrease in cell viability. Second, it can provide quantitative and reproducible results rapidly. Early events leading to intimal hyperplasia, such as SMC migration, can be quantified in as short a time as 3 days. Third, our model reproduces major components of the arterial wall in vitro using a natural collagenous and elastic stroma. Most in vitro models for tissue remodeling use substrates for degradation or cell migration whose structure and composition differ considerably from in vivo conditions. Occasional fibroblasts present in the amnion stroma are killed by a harsh NH$_4$OH treatment, which also inactivates growth factors and other molecules that can affect a variety of endothelial or SMC functions. In contrast, Matrigel, the widely used in vitro reconstituted extracellular matrix, contains several growth factors. The amnion membrane has a dense basement membrane and an abundant collagenous stroma with elastin fibers, which confer great resistance to traction and resilience on the tissue and make it similar to an artery. In contrast, Matrigel lacks the resilience and consistency typical of the arterial wall. Finally, the simplicity of our model allows studying SMC migration without the many confounding factors of in vivo models, such as the presence of blood components, endothelial–white blood cell interactions, and hemodynamic influences. Our in vitro model lacks several factors, including blood cells or cholesterol, that modulate vascular remodeling in vivo. However, these factors can be characterized by adding them exogenously. In our model, the presence of confluent endothelial cells on the basement membrane did not affect SMC migration. Likewise, injury of the endothelial cell monolayer or addition of FGF-2 did not induce SMC migration into the stroma. This observation contrasts with previous findings that endothelial cells affect SMC morphology, proliferation, and protein synthesis. A stimulatory effect of endothelial cells on SMCs has been proposed based on observations that in animal models intimal hyperplasia can occur with an intact endothelium and in humans it can be more severe in areas of intact endothelium relative to partially denuded areas. However, addition of endothelial cell–conditioned medium to or cocultivation of SMC with endothelial cells inhibits SMC proliferation. Endothelial cell–derived FGF-2 acts as a potent mitogen but is not chemotactic for SMC in vitro, although it can stimulate SMC migration in vitro. In our model, neither injury of the endothelial cell monolayer and consequent FGF-2 release nor addition of FGF-2 induced SMC migration. Conversely, in the presence of a PDGF gradient or platelets, SMCs migrated into the amnion stroma, confirming that PDGF and platelets have a strong stimulatory effect on SMC migration. Although we incubated our cultures on the amnion for as long as 10 days in the presence of 50 ng/mL PDGF, we never observed SMC migration through the basement membrane. Possible explanations for this observation include that the amnion basement membrane is thicker and denser than a vessel’s basal lamina, SMC migration through the basal lamina requires additional factors (e.g., cytokines, proteases, and/or proteinase inhibitors) missing in our model, or basement membrane integrity was maintained under our experimental conditions, whereas SMC migration through the basal lamina may require damage of this structure. When we injured the endothelial cell layer with the blunt edge of a forceps, histological examination showed an intact basement membrane. In contrast, arterial injury often entails damage of the basal lamina, as is the case for the widely used balloon injury model of the rat carotid artery or in humans during percutaneous transluminal coronary angioplasty. Several studies have shown the role of TIMP-1 or other anti-MMP agents in controlling SMC migration. Therefore, we used Ad.TIMP-1–transduced SMCs that overexpress TIMP-1 to test the reliability of our model. Consistent with previous findings, upregulation of TIMP-1 expression by adenovirus gene transfer completely inhibited SMC migration into the amnion stroma, showing the reliability of our model. In conclusion, our in vitro model provides a convenient system for studying the individual contribution of several factors to SMC migration and other aspects of vascular remodeling, as well as a rapid, reliable, and inexpensive method for the preliminary characterization of treatments aimed at preventing intimal hyperplasia in vivo.

Acknowledgments
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Fig. I. TIMP-1 expression by adenovirus-transduced hASMC. a) Northern blotting of total RNA from non-transduced, Ad.βgal- or Ad.TIMP-1-transduced hASMC. All the cells express endogenous TIMP-1 mRNA (end.TIMP-1); however, only Ad.TIMP-1-transduced cells show a larger, 1.1 Kb transcript (rec. TIMP-1) consistent with the recombinant TIMP-1 mRNA. Cells infected with a MOI of either 100 pfu/cell (MOI 100) or 10 pfu/cell (MOI 10) express comparable amounts of the recombinant transcript. 18 S rRNA is shown as a control in the lower panel. b) Western blotting analysis of TIMP-1 in medium conditioned by non-transduced, Ad.βgal- or Ad.TIMP-1-transduced hASMC. c) Reverse zymography. Consistent with Northern and Western blotting results, Ad.TIMP-1-transduced hASMC show increased TIMP-1 activity. Purified TIMP-1 and TIMP-2 standards (University Technologies Intl., Inc) are shown as controls in the leftmost lane.
METHODS

Construction of Recombinant, Replication-Deficient Adenoviral Vectors. A full-length human TIMP-1 cDNA (kindly provided by Dr. Henney, Oxford, England) was subcloned into an adenoviral shuttle plasmid (pCMV.Ad), flanked on its 5’ end by the Cytomegalovirus (CMV) promoter. At its 3’ end, a polyadenylation sequence and several hundred nucleotides of Adenovirus type 5 (Ad dl 309) genome lacking the E1A region flanked the insert. The resulting plasmid, pCMVAd.TIMP-1, was linearized with Nru I and cotransfected with the Ad dl 309 viral genome (digested with Cla I and Xba I) into 293 cells stably transfected with the adenoviral E1A and E1B genes (kindly provided by Dr. R. Schneider, NYU School of Medicine, New York, NY). The presence of TIMP-1 cDNA in the recombinant virus particles obtained from lysis plaques was confirmed by polymerase chain reaction and by Southern blotting. The same strategy was used for construction of adenoviral vectors encoding the *E. coli* β-galactosidase (βgal) gene. The resulting recombinant viruses are referred to as Ad.TIMP-1 and Ad.βgal, respectively.

Transduction of Human Aortic Smooth Muscle Cells. Confluent hASMC were washed with PBS and incubated at 37°C for 1 h with 1 ml of PBS supplemented with 2% FBS and containing either no virus (mock infection) or 2 x 10⁸ plaque-forming units (pfu)/ml of Ad.TIMP-1 or Ad.βgal. Under these conditions, the multiplicity of infection (MOI) corresponded to 100 pfu/cell. Transduction efficiency was 12.4 - 24.1% as assessed by β-gal staining. After incubation in complete medium for 24 h, the cells were used for migration assays or harvested for RNA isolation. To obtain conditioned medium for Western blotting or zymography, 24 h after the infection the cells were washed twice with PBS and incubated with 5 ml of serum-free DMEM overnight.
Northern Blotting. Total RNA was extracted from cells with the TRIzol Reagent following the manufacturer’s instructions. Twenty micrograms of total RNA was electrophoresed in a denaturating 1% agarose gel containing 2.2 mol/l formaldehyde and blotted onto a positively charged nylon membrane using a capillary blotting system. The membrane was hybridized with a digoxygenin (DIG)-labeled cDNA probe to TIMP-1 or, as a control, with a DIG-labeled 18S rRNA probe for 12 h at 50°C in DIG Easy Hyb solution (Baxter, Deerfield, IL). The hybridized DIG-labeled cDNA probe was detected by standard chemiluminescence autoradiography with a DIG Detection Kit, BM Chemiluminescence Blotting Substrate (POD; Boehringer Mannheim, Indianapolis, IN).

Western Blotting. Two milliliters of serum-free conditioned medium from transduced or sham-infected hASMC cultures was concentrated to 70 µl using Centricon-10 tubes (Millipore, Bedford, MA) at 4°C. Five to twenty microliters were loaded onto 12% (TIMP-1) SDS/polyacrylamide gels, electrophoresed under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking overnight with 5% (w/v) nonfat dry milk in Tris 20 mM, NaCl 137 mM, HCl 1.0 M, 0.1% Tween 20 pH 7.6 (TBS-T) (blocking solution) the membranes were incubated for 1.5 h at room temperature with a pretested 1:5,000 dilution of rabbit anti-human TIMP-1 antibodies (Chemicon Intl., Inc., Temecula, CA) and subsequently with a 1:5,000 dilution of horseradish peroxidase-labeled donkey anti-rabbit IgG for 1 h (Amersham, Life Technologies, Arlington Heights, MD). Immune complexes were detected with the ECL™ detection system (Amersham, Life Technologies) following the manufacturer’s instructions. The membranes were exposed to autoradiographic films (Hyperfilm MP; Amersham, Life Technologies) for 10 sec to 1 min. Equal loading and transfer of protein
was assessed by staining the blot with 1 % Ponceau Red.

**Reverse Gelatin Zymography.** One microliter of conditioned medium was run under non-reducing conditions in an SDS/12% polyacrylamide gel containing 1 mg/ml of gelatin (Merck, Darmstadt, Germany) and 1 ml of “MMP solution” (University Technologies Intl., Inc). The gel was washed overnight in 100 ml of 50 mmol/l Tris-HCl, pH 7.5 containing 2.5 % Triton X-100 for 30 min at room temperature, followed by two rinses with Tris-HCl alone for 10 min. The gel was incubated in 50 mmol/l Tris-HCl, pH 7.5, 10 mmol/l CaCl$_2$, 0.15 mol/l NaCl, 0.1% Triton X-100, and 0.02% sodium azide at 37$^\circ$ C for 18 h before staining with Coomassie blue and fixation in 10 % glycerol.

**Cell Labeling with $^{125}$I-Iododeoxyuridine ($^{125}$I-dUR).** Subconfluent hASMC were incubated in the presence of 3.0 µCi/ml of $^{125}$I-dUR (2000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) in complete growth medium at 37$^\circ$ C for 24 h. At the end of the incubation, the cultures were washed twice with PBS, trypsinized, and resuspended in 30 ml of medium. The cells were centrifuged at 500 x g for 5 min and resuspended in 1 ml of complete medium. Ten-microliter aliquots of the cell suspension were collected for determination of the radioactivity in a gamma counter (Camberra Packard, Meriden, CT). Cell number was determined with a hemacytometer. Specific labeling ($^{125}$I cpm/cell) was calculated by dividing the $^{125}$I cpm associated with the cell suspension by the number of cells. In sixteen experiments specific labeling for non-transduced hASMC averaged 0.1502 ± 0.049 cpm/cell, for Ad.TIMP-1-transduced cells 0.069 ± 0.026 cpm/cell, and for Ad.βgal-transduced cells 0.144 ± 0.021 cpm/cell. Similar results were obtained with $^{125}$I-dUR-labeled BSMC.
Amnion invasion assay with $^{125}$I-dUR-labeled SMC. were used, the medium was collected in a tube. The membranes were washed twice with PBS and incubated in trypsin/EDTA at 37° C for 20 min. The noninvasive SMC cell layer that remained on the membrane surface was removed by scraping with a rubber policeman, and the membranes were washed three times with αMEM containing 10% DCS. The PBS, trypsin solution, swabs and membranes were collected in separate tubes. The radioactivity associated with the membrane, medium, trypsin, and PBS washings was measured in a gamma counter. The radioactivity associated with the membrane was expressed as percent of the total radioactivity, determined by summing the radioactivity in each tube.

Reference