# Extracellular matrix proteins are potent agonists of human smooth muscle cell migration

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*Purpose:* Extracellular matrix proteins can stimulate smooth muscle cell (SMC) migration by three distinct mechanisms: chemokinesis (nondirected migration in the presence of soluble protein), chemotaxis (directed migration toward soluble protein), and haptotaxis (directed migration toward insoluble, substrate-bound protein). This study investigates the effects of four prevalent extracellular matrix proteins (collagen types I and IV, fibronectin, and laminin), and platelet-derived growth factor (PDGF) on haptotaxis, chemotaxis, and chemokinesis of human SMCs. The role of large guanosine triphosphate-binding proteins (G-proteins) in the signaling mediating these effects is also evaluated.
*Methods:* Human saphenous vein SMCs were used in all migration studies. Chemokinesis, chemotaxis, and haptotaxis to each of the matrix proteins were measured and compared with PDGF through the use of a 48-well microchemotaxis chamber. The role of G-proteins in matrix-induced SMC migration was studied with the modulators of G-protein function, cholera and pertussis toxins.
*Results:* For all matrix proteins the relative strength of the various stimuli for migration was haptotaxis > chemotaxis > chemokinesis ($p < 0.05$). For all three stimuli collagen I and IV produced the most significant migration followed by fibronectin > PDGF-AB > laminin ($p < 0.05$). Pertussis toxin completely inhibited chemotaxis and partially inhibited haptotaxis by laminin but did not affect migration by other matrix proteins, whereas cholera toxin abolished migration in response to all four matrix proteins.
*Conclusion:* Matrix proteins, with the exception of laminin, provide a more significant stimulus for SMC locomotion than does the prototypical agonist, PDGF-AB. Of the three mechanisms by which migration can be stimulated, haptotaxis elicits the most profound effect. The importance of G-proteins as second messengers for migration varies with each matrix protein and with the mechanism of stimulation. (J Vasc Surg 1996;24: 25-33.)

Despite advances in treatment modalities, arteriosclerotic vascular disease remains the leading cause of morbidity and mortality in the United States. The excellent initial rate of success of vascular reconstruction is later diminished by restenosis related to intimal hyperplasia.[1,2] Smooth muscle cell (SMC) migration and proliferation and the production of abundant extracellular matrix (ECM) are necessary steps in this pathologic process.[3] Although the hyperplastic response that follows arterial reconstruction is complex, migration of vascular SMCs from the arterial media to the intima is necessary for the development of an intimal plaque. Animal studies have shown that up to 50% of SMCs in the hyperplastic neointima are not dividing,[4] suggesting that their presence in the subintimal space is related to migration.

SMCs are stimulated to migrate by a plethora of cytokines and growth factors. Platelet-derived growth factor (PDGF) is considered the prototypical agonist of SMC migration. Although a number of these factors including PDGF are released at the time of arterial injury, SMCs are also simultaneously exposed to a variety of matrix proteins.[5] In the uninjured vessel wall the ECM forms the latticework in which medial SMCs reside. After arterial injury oc-

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curs, the kind, quantity, and distribution of these matrix proteins change. Therefore matrix proteins may greatly contribute to the alterations in SMC phenotype that lead to the development of intimal hyperplasia.

The influence of ECM proteins on SMC migration has not been well studied. Experiments to date have focused primarily on attachment of SMC to various matrix-coated surfaces.[6] The relative influence of the most prevalent matrix proteins on SMC migration is not known, nor has the magnitude of the effect of matrix proteins on SMC migration been compared with that of soluble growth factors.

The effect of an agonist on cellular migration can differ significantly depending on the distribution and physical state of the stimulant. Three distinct stimuli of migration have been described. Chemokinesis refers to nondirected cellular migration in response to a soluble factor (in the absence of a concentration gradient). Chemotaxis describes directed migration toward a positive gradient of soluble agonist. Haptotaxis defines directed cellular migration toward an insoluble attractant such as a substrate-bound matrix protein. By far, chemotaxis is the most widely studied of the three mechanisms of migration, and significantly less is known about haptotaxis. Which of these three modalities of stimulation has the greatest influence on SMC migration after arterial injury is currently unknown, although theoretically all three forms may be important.

Matrix proteins promote cellular migration by activating a series of intracellular signaling events. Although these pathways are poorly understood, there is increasing evidence that suggests that heterotrimeric guanosine triphosphate (GTP)-binding proteins (G-proteins) may have an important role in this process.[7] Large G-proteins facilitate communication between cell-surface receptors and a series of intracellular messengers through modification of ion channels and phospholipid metabolism. Studies in neoplastic cell lines demonstrate a role for G-proteins in mediating metastatic migration in response to matrix proteins.[8]

This study was designed to investigate the effects of a series of ECM proteins on human SMC migration. The goals were as follows: (1) to determine the relative strength of the migratory response elicited by four prevalent ECM proteins, (2) to compare the influence of matrix proteins on SMC migration with that of PDGF, (3) to determine which of the three stimuli for migration is most potent, and (4) to define the importance of membrane G-proteins in the signaling cascade that leads to matrix protein-induced migration.

## MATERIAL AND METHODS

**General materials.** Human recombinant PDGF-AB was obtained from Upstate Biotechnologies Inc. (Lake Placid, N. Y.). Bovine fibronectin, Engelbreth-Holm-Swarm mouse sarcoma laminin, calf skin collagen I, human placental collagen IV, and the smooth muscle–specific actin immunostaining kit were obtained from Sigma Chemical Co. (St. Louis, Mo.). Pertussis toxin (PT), cholera toxin (CT), Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline solution (PBS), fetal bovine serum, trypsin-ethylenediaminetetraacetic acid, penicillin/streptomycin/fungizone solution, L-glutamine, and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid were obtained from GIBCO BRL Life Technologies (Gaithersburg, Md.). Polycarbonate 8 μm pore membranes were from Poretics Corp. (Livermore, Calif.).

**Cell culture.** Human SMCs were harvested from explants of remnant portions of saphenous vein intended for aortocoronary or peripheral arterial bypass grafting as previously described.[9] Sections of saphenous vein were opened lengthwise, and the endothelial and adventitial layers were gently removed. Fragments of the medial layer were placed onto tissue culture plates, and outward growing SMCs were harvested and subcultured. Cells were maintained at 37° C and 5% $CO_2$ in DMEM supplemented with 10% fetal bovine serum, 25 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 40 U/ml penicillin G, 40 μg/ml streptomycin, 100 ng/ml amphotericin B, and 4.8 mmol/L L-glutamine. Cells in passages one to five were used for all experimentation. SMC identity was verified by immunostaining with anti-human α-actin antibody and by a characteristic hill-and-valley growth pattern.

**Migration assay.** Before migration experiments were conducted, SMCs were grown to confluence in 100 mm culture dishes and were then made quiescent by incubation in serum-free DMEM for 72 hours. Cells were then washed in PBS, harvested with 0.05% trypsin-ethylenediaminetetraacetic acid, and resuspended in serum-free DMEM.

Migration assays were performed for 4 hours at 37° C in a 48-well microchemotaxis chambers (Neuroprobe, Cabin John, Md.) with upper and lower wells separated by polycarbonate 8 μm pore membranes. For all assays SMCs suspended in serum-free DMEM were seeded at a density of 50,000/well

(6000/mm$^2$) into the upper wells of the chamber. For chemokinesis experiments soluble agonists diluted in serum-free DMEM were introduced into the upper and lower wells. For chemotaxis agonists diluted in DMEM were added to the lower well, and vehicle diluted in DMEM was added to the upper well. In preparation for the haptotaxis experiments membranes were floated on ECM proteins in PBS in the concentrations indicated for 48 hours at 4° C. Membranes were then rinsed with PBS, air dried, and placed into the microchemotaxis chamber with the coated surface facing downward. Control membranes were floated on PBS alone and handled in the same manner. Cell suspension was then added to the upper well, and DMEM alone was added to the lower well of the chamber.

On completion of the assay the membrane was removed from the chamber, fixed in 70% ethyl alcohol at −20° C for 20 minutes, and stained in hematoxylin overnight. The upper side of the membrane was then examined for cell attachment and scraped with a cotton swab; care was taken not to disturb cells that had migrated onto the undersurface. The membrane was then mounted to a microscope slide for viewing, and the migration in each well was assessed by counting the number of cells in five independent high-power fields at 200× magnification. The stimulatory effect of the various agonists was expressed either as the number of cells migrated or as the fold-increase in migration over unstimulated control samples.

**Effect of cholera and pertussis toxins.** In experiments investigating the role of G-proteins in the signaling of matrix-stimulated SMC migration, CT (10 μg/ml) or PT (1 μg/ml) was added to the serum-free DMEM cell suspension in the upper well of the chamber coincident with seeding. Standard chemotaxis and haptotaxis assays were then performed as described previously, and the effect of the toxins on SMC migration was determined as a percent of agonist-stimulated control samples in the absence of toxin (vehicle only).

**Statistical analysis.** Individual experiments were performed in triplicate, and all observations were made with at least three separate cell lines. Values are displayed as the mean ± SD, and statistical comparisons were made with an unpaired Student's *t* test with StatView (Brain Power, Inc., Calabaras, Calif.) software on a personal computer. For comparisons, $p$ values of less than 0.05 were considered significant. Data displayed are representative examples.

## RESULTS

**Concentration-response effects of ECM proteins.** To directly compare the influence of the various ECM proteins on human SMC migration, we first created concentration-response curves for each protein for each of the three stimuli and then used in separate comparative studies the concentrations that produced maximal migration. Accordingly, concentration-response curves were created for collagens I and IV, fibronectin, and laminin for chemokinesis, chemotaxis, and haptotaxis (Fig. 1). For chemokinesis and chemotaxis the concentrations noted indicate the actual concentration of ECM protein to which the SMCs were exposed during the assay. For haptotaxis, concentrations reported refer to the concentration of ECM protein in the PBS solution on which membranes were floated during coating. Because these values do not indicate the actual concentration of ECM protein adsorbed to the membrane surface, they cannot be compared numerically with values for chemokinesis or chemotaxis. In addition, we found no difference in attachment of cells to membranes floated on any of the four matrix proteins or to control membranes floated on PBS alone.

Laminin had no significant chemokinetic effect throughout the range of concentrations tested. For chemotaxis and haptotaxis laminin concentration response curves plateaued once a maximal effect was achieved (Fig. 1). A similar plateau was achieved with chemokinesis, chemotaxis, and haptotaxis in response to fibronectin. The concentration-response curves for the two types of collagen were notably different from those seen with laminin and fibronectin. For chemokinesis, chemotaxis, and haptotaxis, migration diminished in response to concentrations of protein that were higher than those necessary to achieve a maximal response. We have previously observed maximal stimulation of SMC migration to PDGF-AB at a concentration of 5 ng/ml.[9] Concentrations of each protein that provided a maximal response were used in all subsequent comparative assays.

**Comparison of chemokinesis, chemotaxis, and haptotaxis.** The relative strength of the three stimuli for migration (chemokinesis, chemotaxis, and haptotaxis) is unknown. Direct comparisons among chemokinesis, chemotaxis, and haptotaxis were made for each protein (Fig. 2). The relationship was similar for all four ECM proteins. Haptotaxis produced a significantly greater migratory response than did chemotaxis, which in turn was a more potent stimulus for SMC migration than was chemokinesis ($p < 0.05$ for all differences).

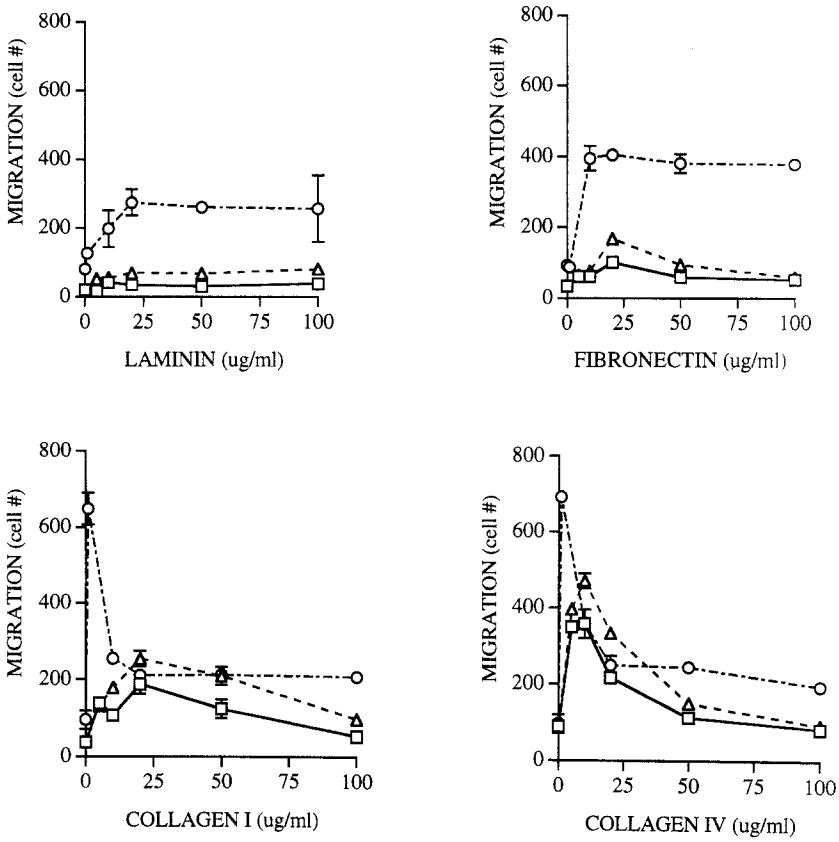


**Fig. 1.** Concentration-response curves for migration of human SMCs to ECM proteins. Human SMCs were stimulated by ECM proteins in indicated concentrations, and chemokinesis (□), chemotaxis (△), and haptotaxis (○) were measured as described in the Material and Methods section. Results are expressed as cells that have migrated ± SD. Experiments were performed in triplicate and repeated in at least three cell lines from different donors. Data from a representative experiment is shown.

**Comparison of ECM proteins.** We assessed the relative effect on SMC migration of the various ECM proteins and PDGF-AB (Fig. 3). Comparisons with PDGF-AB could be made only for chemokinesis and chemotaxis. In a representative experiment displayed in Fig. 3 collagen IV produced a more potent response than did collagen I for all three forms of stimulation ($p < 0.05$). However, this relationship was not consistent, and in some experiments collagen I and collagen IV were equally potent. Both types of collagen consistently produced more migration than fibronectin ($p < 0.05$), which had a stronger influence on migration than did laminin ($p < 0.05$). The chemokinetic and chemotactic effect of PDGF-AB was intermediate between laminin and fibronectin ($p < 0.05$). With the exception of laminin the ECM proteins induced a far more significant migratory response than did the prototypical agonist of migration, PDGF-AB.

**Role of GTP-binding proteins.** We hypothesized that differences in cell locomotion produced by the various matrix proteins might be related to differential activation of cellular signaling pathways. A significant role for the large G-proteins in ECM-induced movement of tumor cells has been described.[7,8] To evaluate the importance of G-proteins in ECM-induced SMC migration, we used two agents: CT, which adenosine diphosphate-ribosylates the G-protein stimulatory subunit ($G_s$), resulting in persistent activation of adenylate cyclase and production of cyclic-adenosine monophosphate (cAMP), and PT, which adenosine diphosphate-ribosylates an inhibitory subunit ($G_i$), preventing its interaction with membrane receptors.[10] SMC and toxins were introduced simultaneously. CT (10 μg/ml) profoundly inhibited SMC migration in response to all agonists (Fig. 4, $p < 0.05$ for all comparisons) and even eliminated baseline migration in unstimulated

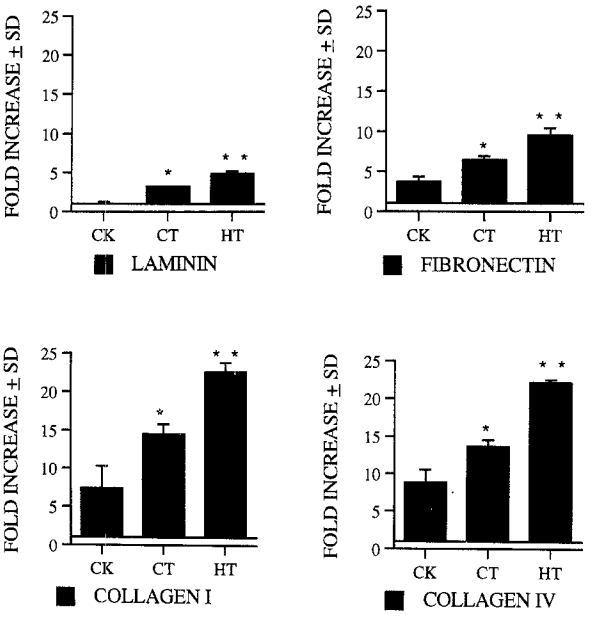


**Fig. 2.** Comparison of migratory effects of chemokinesis, chemotaxis, and haptotaxis. Human SMCs were stimulated with ECM proteins in concentrations that provided maximal chemokinesis *(CK)*, chemotaxis *(CT)*, and haptotaxis *(HT)*. Results are expressed as fold-increase in migration versus unstimulated control ± SD. Experiments were performed in triplicate and repeated in at least three cell lines from different donors. Data from a representative experiment is shown ($p < 0.05$, *CK compared with CT; **CT compared with HT).

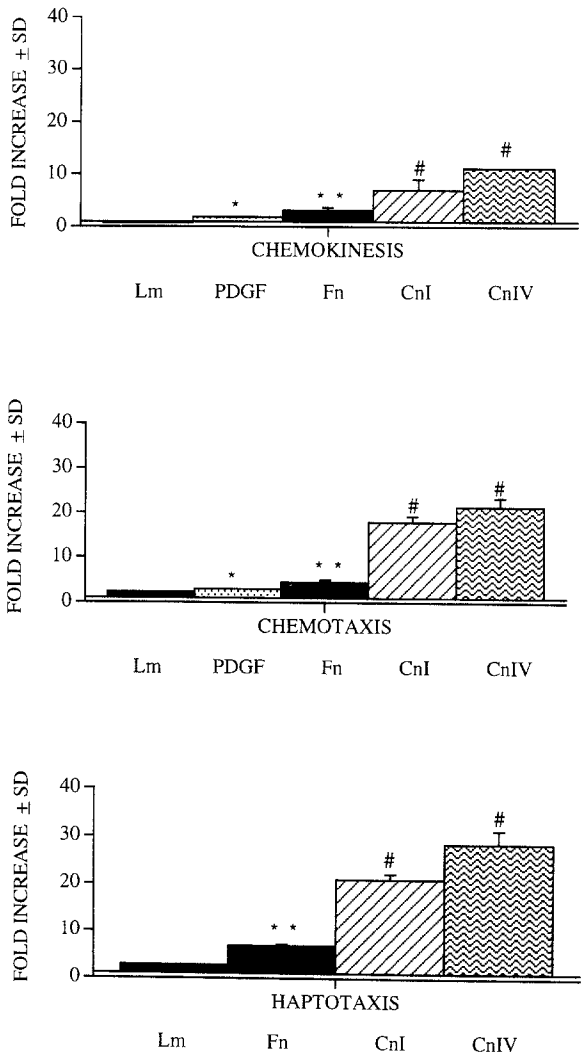


**Fig. 3.** Comparison of migratory effects of laminin, fibronectin, and collagen I and IV. Migration of human SMCs exposed to laminin *(Lm)*, fibronectin *(Fn)*, collagen type I *(CnI)*, and collagen type IV *(CnIV)* with concentrations for each that produced maximal effect. PDGF-AB was also used in comparisons of chemokinesis and chemotaxis. Results are expressed as fold-increase in migration versus unstimulated control ± SD. Experiments were performed in triplicate and repeated in at least three cell lines from different donors. Data from a representative experiment is shown ($p < 0.05$, *PDGF compared with Lm, **Fn compared with PDGF or Lm, # Cn I or IV compared with Fn).

cells (data not shown) in the control group. PT (1 μg/ml) had no effect on chemotaxis or haptotaxis to fibronectin, collagen I, or collagen IV. However, PT partially inhibited haptotaxis (26% ± 10%) and completely eliminated chemotaxis to laminin (Fig. 4, $p < 0.05$ for both differences).

## DISCUSSION

At the time of arterial injury SMCs are simultaneously exposed to multiple growth factors and to a host of extracellular matrix proteins. The effect of the former on SMC migration has been thoroughly evaluated; however, less is known about the influence of matrix proteins on SMC locomotion. This study evaluates the effect of four predominant extracellular matrix proteins on migration of human vascular SMCs. We chose to study SMCs derived from human saphenous vein because of the relevance of these cells to stenoses that develop after autogenous arterial reconstruction.

Evaluation of the effect of matrix proteins on SMC migration is potentially complex, because the stimulus for migration can vary with the solubility of the agonist and its location relative to the affected cell. Three different forms of migration have been defined. If a gradient of soluble agonist does not exist, SMC will be stimulated to migrate in a random fashion (chemokinesis). Directed migration toward a soluble attractant (growth factor or matrix protein) is termed chemotaxis, and haptotaxis defines migration of cells toward a gradient provided by an insoluble protein

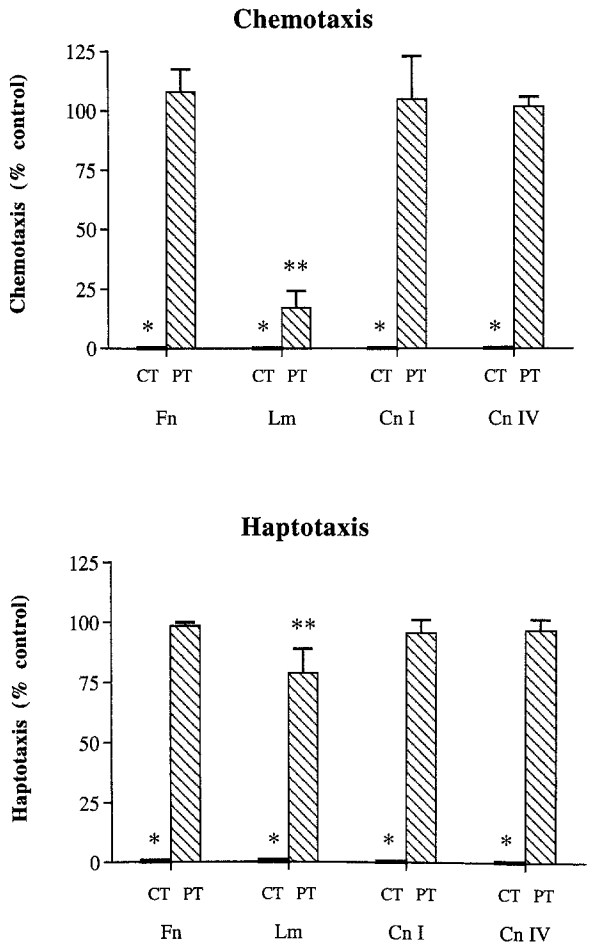


**Fig. 4.** Effect of cholera and pertussis toxins on chemotaxis and haptotaxis. Human SMCs, coincubated with CT (10 μg/ml) or PT (1 μg/ml), were exposed to either chemotactic or haptotactic stimulus of fibronectin *(Fn)*, laminin *(Lm)*, collagen type I *(CnI)*, or collagen type IV *(CnIV)*. Results are expressed as percent of control (vehicle only) chemotaxis/haptotaxis to each of the proteins ± SD. Experiments were performed in triplicate and repeated in at least three cell lines from different donors. Data from a representative experiment is shown ($p < 0.05$, *CT compared with control; **PT compared with control).

(ECM protein). All three stimuli could potentially influence the vascular SMCs during the remodeling process that follows an arterial injury.[3] Autocrine release of growth factors such as PDGF might stimulate SMC migration via chemokinesis. Circulating blood products (both growth factors and soluble matrix proteins) present at the luminal surface of an injured artery can provide a directional stimulus for SMC migration (chemotaxis). These same matrix proteins, once substrate-bound, or insoluble matrix proteins that make up the basement membrane, may also provide a haptotactic stimulus for migration. To evaluate these various mechanisms of migration we used four agonists, fibronectin, laminin, and collagen types I and IV, all of which are prominent components of either the extracellular matrix or basement membrane.

In an initial series of studies we determined the concentration of each matrix protein that for each migratory stimulus resulted in maximal cell movement. With these concentrations we were able to make valid comparisons between the migratory responses elicited by each protein and each stimulus. Migration in response to fibronectin and laminin peaked at intermediate concentrations and then plateaued when SMCs were stimulated with higher concentrations of these proteins. Presumably a concentration was reached where integrin receptors were saturated, preventing higher concentrations of these matrix proteins from producing any additional effect. In contrast, for collagens I and IV maximal migration was produced by intermediate concentrations of protein, and migration diminished when higher concentrations of collagen were used. Similar observations were made by DiMilla et al.,[11] who found that increasing concentrations of collagen IV enhanced SMC attachment but diminished the ability of the SMCs to migrate. They hypothesized that the ability of a cell to migrate is inversely related to its potential to attach. It is unclear why the concentration response curves for the collagens and laminin and fibronectin differ; however, this observation provides additional evidence that the effect of matrix components on SMC migration is specific for each protein.

The strongest migratory response followed stimulation of SMC by the two collagens; fibronectin was intermediate in strength, and laminin provided the least potent stimulus for migration. This hierarchy was identical for chemokinesis, chemotaxis, and haptotaxis. Previous studies have revealed differential effects of matrix proteins on SMC phenotype. Fibronectin has been shown to convert SMC from a differentiated contractile state to a synthetic and proliferative phenotype.[12] Our observation of a strong stimulatory effect of fibronectin on SMC migration is consistent with this ability of fibronectin to transform vascular SMCs. Both forms of collagen were also potent agonists of migration. Even laminin stimulated migration of human SMC, albeit to a much lesser degree. However, laminin and the collagens have been shown by others to inactivate SMC and promote differentiation.[12] The reason for these discrepant findings is unclear; however, the effect of

these matrix proteins on SMC migration was reproducible with SMC derived from different passages and from varying donors.

We compared the effect of matrix proteins on SMC migration with that of the soluble growth factor PDGF-AB. We chose PDGF-AB because studies have shown that this is the predominant isoform found in human platelets.[13] With the exception of laminin matrix proteins were consistently more potent agonists of SMC migration than PDGF-AB. Although the magnitude of the response varied with each cell line tested, the chemotactic response of matrix proteins ranged from 4.5- to 21-fold that of cells in the control group, whereas PDGF-AB consistently produced a two- to threefold increase in SMC chemotaxis. In separate experiments we and others have observed that PDGF-BB produces a slightly greater (20% to 30%) migratory response than does PDGF-AB (data not shown). However, even the effect of PDGF-BB on SMC migration is much less than that observed with the two collagens. We have previously shown other soluble growth factors such as epidermal growth factor and basic fibroblast growth factor to be less potent agonists of SMC migration than PDGF-AB.[9] These findings suggest that matrix proteins may be the predominant stimulus for the SMC migration that occurs after arterial injury.

Of the three stimuli for migration we consistently found that the most profound effect on migration occurred when SMCs were stimulated by a gradient of insoluble matrix protein (haptotaxis). This observation was consistent for all four matrix proteins, and the response to haptotaxis was on average 33% greater than the response of SMC to chemotaxis for the same agonist. Haptotaxis has been best studied in models of neoplasia. Because extracellular matrix proteins are most often present in vivo in insoluble form, haptotaxis is thought to play a significant role in the invasion and metastatic spread of malignant tumors. From these studies it appears that haptotaxis and chemotaxis are not simply extensions of one another but completely separate processes.[14] In fact, recent studies show that the signaling pathways responsible for these different mechanisms of stimulation may be distinct. In the case of thrombospondin, haptotaxis and chemotaxis are mediated by completely separate peptide domains.[15]

Because activation of G-proteins has been found to be necessary for matrix-induced chemotaxis and haptotaxis of several tumor cell lines, we evaluated the importance of G-proteins in matrix-driven migration of human SMCs. CT, which adenosine diphosphate ribosylates and leads to persistent activation of $G_s$, dramatically inhibited migration to all matrix proteins and migration of cells in the control group. These effects simply may be related to the increased levels of cAMP that are produced by persistent activation of $G_s$.[10] This hypothesis is supported by previous observations that stimulation of SMC with direct agonists of cAMP such as forskolin and 8-bromo-cAMP inhibited migration.[16]

PT, which inhibits at least three subtypes of $G_i$ ($G_{i1}$, $G_{i2}$, and $G_{i3}$) by uncoupling these proteins from their receptors,[10] had varying effects on SMC migration that differed with each matrix protein and with the stimulus for migration. Activation of $G_i$ appears to be essential for chemotaxis by laminin and partially necessary for haptotaxis by this same protein. Neither chemotaxis nor haptotaxis to the collagens or fibronectin was influenced by inhibition of $G_i$. From these data we conclude that signaling pathways for the various matrix proteins differ and that at least for laminin signaling pathways for the various stimuli for migration (haptotaxis versus chemotaxis) may also be distinct. Aznavoorian et al.[17] also found a differential role for G-proteins in migration of a melanoma cell line that was dependent on the matrix protein and the stimulus for migration.

Our findings show that the ECM has a profound influence on SMC migration and that the magnitude of this effect is significantly greater than that of previously studied growth factors. Collagen types I and IV, both of which are plentiful in the vessel wall, are the most potent agonists of migration, with laminin having the least significant influence. Stimulation through haptotaxis has a much greater effect on cellular motility than either chemotaxis or chemokinesis. The signaling pathways used by ECM proteins may vary with each specific protein and with each mechanism of stimulation.

Migration is an essential component of the intimal hyperplastic process. Therefore inhibition of migration might prevent the formation of hyperplastic lesions that so often complicate vascular reconstruction. We have found that SMC migration is influenced by matrix proteins to an even greater degree than by growth factors such as PDGF. These findings suggest that attention should be focused on designing inhibitors that effect SMC/matrix protein interactions.

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## DISCUSSION

**Dr. Allan D. Callow** (Boston, Mass.). I think this is a splendid article for many reasons, and I congratulate you, Dr. Nelson, and your colleagues for calling attention to those methods and phenomena that probably will lead to the solution of the problem of restenosis. I admire your courage. You have taken on so many different mechanisms and several proteins.

We know that extracellular matrix does modify the phenotype of the SMC. Has the phenotype of the vascular SMC already been altered in the course of your processing? If it has been altered, then it is now presumably secretory rather than contractile. Therefore it may be contributing to the source of the agonist, that is, the collagen that you say is so effective here.

Second, what type of collagen do you have? Is it an active? Is it cross-linked? Is it denatured? What was the source of your collagen?

And third, I am curious why you chose the collagen you did. You did not study collagen type III, nortropoelastin, which are prominent in these phenomena.

**Dr. Peter R. Nelson** (Boston, Mass.). The first question you referred to points to the limitation of using cultured cell model. We use human vascular SMCs from saphenous vein. They are extracted by explant from remnant portions of saphenous vein left over from aortocoronary or peripheral vascular procedures.

**Dr. Callow.** Could you tell us how you extracted them? My point is, what is the stage, the status of the SMC by the time you have obtained it and put it into your model?

**Dr. Nelson.** We extract them by a standard explantation technique where the adventitial and intimal layers are scraped away and then the remaining medial portion is placed into a dish and allowed for outward growth of SMCs. Once the cells have grown out to a sufficient area, we harvest those cells with trypsin and potentiate cultures from there on out.

The question of whether we have altered cells by definition that we have now cultured them and whether they are in a proliferative versus a quiescent phase, two things come to mind. First of all, our cells are made quiescent before the study so that we starve the cells for 72 hours in serum-free media, so they are both confluent in a dish and are starved for 72 hours, and we have actually done previous cell cycle analysis in the laboratory to show that those are truly quiescent cells at the time of initiating the experiment.

As to the next question, which was the source of the collagen, we do not get our collagen from any special source. It is commercially available collagen from Sigma Corporation, who provides it to us, so there is nothing special about our collagen preparation.

In terms of our choice of proteins that we studied, there was no specific reason not to include collagen type III. There have been a number of studies with any one of the

proteins or an occasional comparison study of two of the proteins, and we compiled those data to arrive at these four proteins to try to in some ways produce an overview comparison study.

**Dr. Michael S. Conte** (New Haven, Conn.). I have two questions. First, as you may know, in a number of recent in vivo studies, interference with cell cycle–regulating proteins has had broad phenotypic effects on vascular smooth-muscle cells including migration. I wonder if you could comment on how you synchronized your cells in relation to the cell cycle. Did you deprive them of serum before you started your experiments?

And the second question. I may have missed some of the method, but could you comment on a role for PDGF? Certainly in vivo PDGF does not act alone in the absence of matrix proteins. Did you look at the combination of PDGF with matrix proteins, which might be more relevant to the clinical situation?

**Dr. Nelson.** The serum starvation question I already addressed to a point, but to stress, what we do is, the cells are grown in culture, and we use passage cells from passage one through passage four, so these are early passage cells, and they are serum-starved for 72 hours. Cell cycle analysis has shown that even before 72 hours these cells are quiescent, and so that 72 hours may be even a little bit excessive, but that has been our practice.

In terms of the PDGF and extracellular matrix combination question, that by chance is a very interesting question that I have been looking at more recently in my studies, and although I do not have data to present here, I hope at some coming conference I may be able to bring some interesting data for you. Preliminarily speaking, it seems that combinations of extracellular matrix and PDGF produce not only an enhanced response, and what I mean by that is the response that we get is more than a simple additive response between proteins, so if you took the effect from any of our data here for the matrix proteins and you added to that the magnitude of the PDGF effect, in combining both, coincubating both proteins in the experiments, we have been able to show that you get a synergistic effect, so the resultant migration is higher than what you would normally anticipate.

**Dr. Bauer E. Sumpio** (New Haven, Conn.). Very interesting study. I would like to make a quick comment so I can ask two quick questions.

The comment here is, I guess you are alluding to it in this study, and that is the interface that occurs between the substrate and cell. It is clear from seminal work done 5 years ago by Joe Madri and also by Folkman that the substrate on which these cells are cultured will influence their phenotype, and in this case you have shown the migration. Right at that interface there is an important structures. It is called the focal adhesion plaque, and this is really where the cell makes contact with the substrates, and right in that focal adhesion plaque are so-called integrins, which bind specifically to certain substances on the matrix and in turn lead to sort of the outside/inside signaling mechanism that you have alluded to in your guanosine triphosphate analysis. So the question I have is, does the integrin pattern in SMCs correlate with the findings that you found in terms of their migration on the different types of substrates, that is, there are specific integrins that bind to laminin and fibronectin, and hence could one predict on the basis of the density and distribution of these integrins that you would see this pattern of migration?

The second question is, in terms of the signaling aspect that you are alluding to with your guanosine triphosphate, are you alluding to the small guanosine triphosphate-binding protein rho, which is part of the cascade that occurs from these focal adhesion plaques?

**Dr. Nelson.** With regard to the first question, there has been a fairly large group of work done primarily in neoplastic cells again but also in vascular cells looking at specifically the integrins and the combinations of the various components composing the integrin receptor in terms of the specificity for the various matrix surfaces. We ourselves in our laboratory have not looked specifically at the integrins and their role in terms of the attachment to and subsequent signaling of the matrix proteins. The focal adhesion complex that you mentioned is actually an interest in our laboratory. One of the other investigators has been looking at the focal adhesion complex, and the plans at this point are to combine some of my interests and some of their interests and try to sort that out for a vascular cell model.

In terms of the small G protein rho, that is not what I am referring to in terms of my study. What we are looking at are the larger G proteins that are essentially known to transfer the signal from membrane receptors to subsequent signaling mechanisms. One particularly is the cyclic AMP or adenylate cyclase pathway. In those pertussis toxin inhibits the inhibitory subunit or inhibitory subtype of those large G proteins, and cholera toxin inhibits the stimulatory subtype. Obviously that is a larger discussion, but essentially we are looking at the large G proteins. Others in our laboratory are also looking at rho, and our laboratory feels very strongly that rho plays a very significant role in the signaling mechanism specifically for SMC migration.