Plasminogen Activator Expression in Rat Arterial Smooth Muscle Cells Depends on Their Phenotype and Is Modulated by Cytokines

Marie-Luce Bochaton-Piallat, Giulio Gabbiani, Michael S. Pepper

Abstract—Cultured rat aortic smooth muscle cells (SMCs) exhibit at least 2 phenotypic variants: (1) a spindle-shaped phenotype, obtained from normal adult media, and (2) an epithelioid phenotype, obtained from intimal thickening 15 days after endothelial injury. Both phenotypes can be cloned from each location, with normal media yielding a majority of spindle-shaped clones and intimal thickening yielding a majority of epithelioid clones. These findings suggest that intimal thickening develops essentially from a subpopulation of medial SMCs exhibiting epithelioid features in vitro. Using zymographic and Northern blot analyses, we have studied plasminogen activator (PA) expression by these SMCs. Our results show that epithelioid SMCs, cultured as whole SMC populations or as clones, display higher PA activity than do spindle-shaped SMCs, irrespective of their origin. This is mainly due to differences in the expression of tissue PA and, to a lesser extent, urokinase PA and is accompanied by a decrease in PA inhibitor 1. Tissue PA activity is increased by basic fibroblast growth factor and platelet-derived growth factor-BB, particularly in epithelioid SMCs. Taken together, these results indicate that SMCs are heterogeneous with respect to their proteolytic profile, at least as far as the PA system is concerned. Proteolytic activity of the different SMC populations is modulated by cytokines that play a role in intimal thickening. Our results are in agreement with the suggestion that epithelioid SMCs are mainly responsible for intimal thickening. (Circ Res. 1998;82:1086-1093.)

Key Words: atherosclerosis n tissue plasminogen activator n basic fibroblast growth factor n platelet-derived growth factor

Smooth muscle cell replication and migration from the media into the intima are essential processes in the development of the atheromatous plaque (for review, see Reference 1). Several laboratories, including our own, have suggested that SMCs from the healthy arterial wall are heterogeneous in nature and that a subset of medial SMCs is particularly prone to migrate and accumulate within the intima.2–4 SMC heterogeneity has been studied by isolating cell populations from different locations or from the same location at different ages.2,4–6,8 Two main SMC phenotypes have been described, mostly in the rat: (1) a spindle-shaped phenotype, with the classic "hill-and-valley" growth pattern, usually obtained from the normal adult media, and (2) an epithelioid phenotype with cells growing as a monolayer, which is isolated from the intimal thickening induced 15 days after endothelial injury.2,4 When SMCs are cloned from either source, both phenotypes are retrieved, and these remain stable during culture conditions.9 However, the proportion of clones with these phenotypes is different, with normal media yielding a majority of spindle-shaped clones and intimal thickening yielding a majority of epithelioid clones. Epithelioid SMCs, cultured as whole or clonal cell populations, are capable of growing in the absence of serum and exhibit higher migratory activity than do spindle-shaped cells.2,3,9 Taken together, these findings suggest that intimal thickening develops essentially from a subpopulation of medial SMCs exhibiting epithelioid features in vitro.

Cell migration is a complex process that includes the disruption of anatomic barriers such as basement membranes. During tumor invasion, it has been clearly shown that both tumor and stromal cells express enzymes that degrade components of the extracellular matrix. Many of the relevant enzymes belong to one of two families: the serine proteases, in particular the PA-plasmin system, and the matrix metalloproteinases. uPA and tPA are the principal activators of plasminogen, the zymogen from which plasmin is derived. uPA (or, more precisely, its inactive precursor pro-uPA) is secreted as a soluble protein that binds with high affinity to a specific cell surface receptor. Plasmin is a protease of trypsin specificity, which, either directly or indirectly through the activation of certain latent matrix metalloproteinases, hydrolyzes many extracellular proteins. Plasminogen and plasmin also associate with plasma membranes, and colocalization of uPA and plasminogen on the cell surface increases the
efficiency of plasminogen activation and subsequent plasmin-dependent proteolysis. The existence of multiple specific physiological inhibitors of both plasmin (ie, α2-antiplasmin) and PA (ie, PAI-1 and PAI-2) provides additional points of regulation along this protease cascade (for review, see References 10 to 12).

The purpose of the present study was to evaluate PA activity in spindle-shaped and epithelioid SMCs, cultured as whole or clonal cell populations. We also studied the effect of cytokines such as bFGF, PDGF-BB, and TGF-β1 on PA activity, since these cytokines are known to play a role in the repair phenomena that follow arterial injury (for review, see Reference 1). Our results show that epithelioid cells display higher PA activity than do spindle-shaped cells. This is mainly due to increased expression of tPA and, to a lesser extent, to decreased expression of PAI-1. bFGF and PDGF-BB significantly increase tPA activity in whole or clonal cell populations, irrespective of their phenotype, whereas TGF-β1 has little effect. uPA is barely affected by these cytokines, whereas PAI-1 is increased by all three.

**Materials and Methods**

**Cell Culture**

SMCs were isolated from adult rat (6 to 8 weeks old) thoracic aortic media and from 15-day-old intimal thickenings produced by mechanically removing the endothelium of the thoracic aorta by means of an inflated embolectomy catheter. Cells were grown in high-glucose DMEM (GIBCO) containing penicillin (100 IU/mL, GIBCO) and streptomycin (100 IU/mL, GIBCO) supplemented with 10% FCS (Seromed). Ten sets of whole SMC populations (5 from normal media and 5 from intimal thickening) were studied between the 5th and the 8th passages.

SMCs were cloned by limiting dilution (0.5 cells per well) during primary culture into FCS-coated 96-well plates in DMEM containing 20% FCS. Cloned SMCs were expanded up to the 3rd passage under the same conditions; they were then cultured in the same way as the whole SMC populations. Overall, 10 SMC clones were studied: 5 spindle-shaped clones (3 from normal media and 2 from intimal thickening) and 5 epithelioid clones (3 from normal media and 2 from intimal thickening). Clones were used between the 7th and the 12th passages.

**Cytokine Treatment**

Whole SMC populations and SMC clones were treated with the following cytokines in DMEM plus 10% FCS, unless otherwise stated: bFGF (kindly provided by Dr P. Sarmientos, Farmitalia Carlo Erba, Milan, Italy) at 10 ng/mL, PDGF-BB (kindly provided by Dr G. Pierce, Amgen, Thousand Oaks, Calif) at 30 ng/mL, and TGF-β1 (R&D Systems, Abingdon, UK) at 1 ng/mL. Over the time course of the experiments (15 hours), bFGF, PDGF-BB, and TGF-β1 did not change the cell morphology of either whole SMC populations or clones.

**Plaque Assay and Zymographic and Reverse Zymographic Assays**

Cells were seeded at the density stated below into 35-mm tissue culture dishes in DMEM plus 10% FCS. Twenty-four hours after the last medium change, culture dishes were washed twice with serum-free DMEM. Cytokines were then added for 15 hours at the indicated concentration in 1.5 mL serum-free DMEM containing Trasylol (200 KIU/mL, Bayer).

For the plaque assay, cells were seeded at 5×10^4 cells per dish, and the cultures were overlaid 24 hours later essentially as previously described. Briefly, monolayers were washed twice with PBS containing acid-treated BSA (1 mg/mL) and overlaid with a mixture containing 2% instant nonfat dry milk, 0.8% agar, and plasminogen (40 μg/mL) in DMEM. The plates were incubated at 37°C for 60 to 120 minutes and photographed under dark-field illumination.

For zymographic and reverse zymographic assays, cells were seeded at 10^5 cells per dish and grown to subconfluence, and culture media and cell lysates were analyzed as previously described. For reverse zymographic assays, samples were preincubated with 0.5% SDS and 0.5% β-mercaptoethanol for 1 hour at 37°C to neutralize PA activity. Aliquots (20 μL) were subjected to SDS-PAGE and zymographic analysis using a casein- and plasminogen-containing substrate gel. Human urokinase (0.05 U/mL, Serono) was added to substrate gels for reverse zymographic analysis. Zymograms and reverse zymograms were photographed under dark-field illumination.

In each experiment, cell numbers were determined in a duplicate series of dishes incubated in parallel; samples were then analyzed on the basis of cell number equivalents. The levels of tPA, uPA, and PAI-1 expression were evaluated by scanning photographs with an Arcus II scanner (Agfa). Results are shown as the sum of pixel values of the area of cleft lysis (in the case of tPA or uPA) and of inhibition of lysis (in the case of PAI-1). For tPA expression, the sum of the signals for cellular, secreted, and PAI-1–complexed tPA were all taken into account in this assessment.

**RNA Preparation, In Vitro Transcription, and Northern Blot Hybridization**

Twenty-four hours after the last medium change, SMCs cultured in 100-mm culture dishes were treated with cytokines as indicated. Total cellular RNA was prepared 4 or 15 hours later as previously described. RNA preparation, Northern blots, in vitro transcription, hybridization, and posthybridization washes were as previously described.

For Northern blots, 5 μg of total cellular RNA was loaded per lane. 32P-labeled cRNA probes were prepared from rat CRBP cDNA clones (kindly provided by Drs J. Eriksen and P. Kristensen) cDNA clones as previously described. Films were scanned as described above.

**Statistical Analysis**

Results are shown as mean±SEM. For statistical evaluation, the results were analyzed by the Mann-Whitney test. Differences were considered statistically significant at values of *P*<0.05.

**Results**

**Whole SMC Populations**

**PA Expression**

PA activity of whole SMC populations cultured from the normal media (spindle morphology) or from intimal thickening (epithelioid morphology) was first compared by means of the plaque assay. Studies were performed with early-passage cells (passages 5 to 8) just before they reached confluence.
Under these conditions, plasminogen-dependent zones of casein lysis (as seen by dark-field illumination), which form around individual cells, were barely detectable in the SMC population cultured from normal media, whereas they were clearly visible in the IT SMC population. This indicates that the SMC population cultured from intimal thickening exhibits higher net PA activity compared with cells from the normal media (Figure 1).

In order to identify the enzymes responsible for this activity, a zymographic analysis was performed on cell extracts and culture supernatants of the different populations. tPA and uPA activities were detected in cellular extracts, each as a single band, and secreted tPA was revealed in culture supernatant as 2 bands, with the higher band corresponding to tPA complexed with PAI-1 (Figure 2). As described in "Materials and Methods," the levels of tPA, uPA, and PAI-1 expression were evaluated by densitometry. Results showed that PA activity detected in the whole SMC population cultured from intimal thickening was mainly due to high tPA activity, which was present in cell extracts as well as in culture supernatants (Figures 2 and 3). In contrast, in the whole SMC population cultured from normal media, tPA activity was low. As shown by reverse zymography, PAI-1 was negative in the majority of experiments: when it was detected, PAI-1 was exclusively expressed in culture supernatant and was significantly lower in SMCs cultured from intimal thickening than in SMCs cultured from the normal media (Figures 2 and 3). uPA activity was weakly expressed in both populations and was detectable only after a long incubation of the zymograms; however, it was significantly higher in the whole SMC population cultured from intimal thickening compared with the whole SMC population cultured from normal media (P<0.05). Although tPA activity did not vary with cell passage number and the degree of confluence, uPA disappeared in late passages and decreased after confluence (data not shown).

PAI-1 mRNA levels paralleled zymographic activity in whole SMC populations derived from normal media and intimal thickening (Figure 4). uPA and uPAR mRNAs were not detected in either cell type. PAI-1 mRNA expression was significantly higher in SMCs derived from intimal thickening than in SMCs derived from normal media (the same results were obtained in each of 3 experiments).

**Effect of Cytokines on PA Expression**

Using the PA plaque assay, we observed that bFGF increased the number and size of pericellular plasminogen-dependent zones of casein lysis in whole SMC populations derived from both normal media and intimal thickening (Figure 1). How-
ever, in agreement with differences in basal levels of expression, the bFGF-induced increase was greater in whole SMC populations derived from intimal thickening than from normal media (Figure 1).

Zymographic analysis revealed that bFGF and PDGF-BB were able to substantially increase tPA activity in the whole SMC population derived from intimal thickening and, to a lesser extent, in the whole SMC population derived from normal media, in cell extracts as well as in culture supernatants (Figures 2 and 3). In contrast, both cytokines significantly decreased uPA activity in whole SMC populations derived from normal media and intimal thickening (P<0.05, Figure 2). PAI-1 expression was increased by bFGF and PDGF-BB in both populations (Figures 2 and 3). Although TGF-β1 had no effect on the PAs, it increased PAI-1 in both cell types (Figures 2 and 3). Effects of the different cytokines on whole SMC populations are summarized in the Table.

Northern Blot analysis (Figure 4) revealed that tPA mRNA expression was increased by bFGF and PDGF-BB in the whole SMC population derived from intimal thickening. In contrast, these cytokines barely affected tPA mRNA expression in the whole SMC population derived from normal media. uPA mRNA was not detected under any conditions. All cytokines tested induced the expression of uPAR mRNA.
when cells were treated for 4 hours. bFGF and PDGF-BB were particularly effective on the whole SMC population derived from intimal thickening. In cells treated for 15 hours, uPAR signals had disappeared (data not shown). Similarly, all cytokines tested increased PAI-1 mRNA expression in both SMC populations, and this was more prominent at 4 hours than at 15 hours after treatment.

SMC Clones

**PA Expression**
The PA activity of 10 different spindle-shaped and epithelioid clones derived either from normal media or from intimal thickening was compared by means of zymographic analysis on cell extracts and culture supernatants (Figures 5 and 6). tPA activity was undetectable or weak in spindle-shaped clones, irrespective of their origin. In contrast, tPA activity was very high in cell extracts as well as in culture supernatants of epithelioid clones derived from either location. Generally, uPA activity was weakly present in all clones irrespective of their phenotype and origin, except in 2 epithelioid clones derived from the intimal thickening. It is noteworthy that uPA activity varied from one clone to another depending on the degree of confluence. Reverse zymograms showed that PAI-1 activity was weakly expressed in culture supernatants of spindle-shaped and epithelioid clones irrespective of their origin. Therefore, with respect to tPA activity, spindle-shaped and epithelioid clones, irrespective of their origin, behaved similar to whole SMC populations derived from normal media and intimal thickening, respectively (Figure 3).

**Effect of Cytokines on PA Expression**
Generally, the effect of the different cytokines on tPA activity was similar on different clones having the same morphological appearance, irrespective of their origin (Table and Figures 5 and 6). bFGF and PDGF-BB greatly increased tPA activity, particularly in epithelioid clones. IPA activity was not affected by TGF-β1 in the 10 clones studied, except in 1 epithelioid clone derived from intimal thickening, in which it was decreased. uPA activity was decreased by bFGF in all cases. uPA activity was generally unaffected by PDGF-BB and TGF-β1, although there were some exceptions: out of 10 clones studied, it was increased by PDGF-BB in 1 epithelioid clone derived from normal media and by TGF-β1 in 2 spindle-shaped clones derived from normal media and intimal thickening. PAI-1 activity was increased by the 3 cytokines tested in all types of clones. Therefore, the clones respond similarly to the 3 cytokines, independent of their phenotype and origin, with respect to tPA, uPA, and PAI-1 activities (Table and Figure 3). With the exception of the action of PDGF-BB on uPA activity, clones behaved in a manner similar to that of the whole SMC populations having the same phenotypic features (Table).

**Discussion**
It is becoming increasingly accepted that the normal arterial media contains several SMC subsets that are likely to play
distinct roles in the repair phenomena that follow arterial injury.\(^1\) However, much work remains to be done to characterize these subsets in terms of functional markers. To address this problem, we have isolated SMC clones from the normal rat aortic media and intimal thickening 15 days after endothelial injury and have shown that these two locations yield the same clonal populations, but in different proportions.\(^9\) Additional work has shown that spindle-shaped and epithelioid clones differentially express certain proteins, such as CRBP-1 and cytokeratin 8, which have been suggested to be markers for epithelioid SMCs.\(^17\) CRBP-1 is expressed in most SMCs derived from intimal thickening, which develops in the rat aorta after endothelial injury, suggesting that the epithelioid SMCs are mainly responsible for the formation of this lesion.\(^17\)

By focusing on the PA/plasmin system, in the present work we have assessed the proteolytic activity of spindle-shaped and epithelioid whole SMC populations and clones. Our results indicate that epithelioid SMCs cultured from intimal thickening 15 days after endothelial injury exhibit higher proteolytic activity than do spindle-shaped SMCs isolated from normal media. This is mainly due to increased expression of tPA and, to a lesser extent, uPA. Additionally, PAI-1, which is a physiological PA inhibitor, is more weakly expressed in epithelioid cells than in spindle-shaped cells. Similar results were obtained when epithelioid clones were compared with spindle-shaped clones, irrespective of their origin, as far as tPA and PAI-1 expression is concerned. uPA and PAI-1 activities were barely detectable in all clones studied, except in 2 epithelioid clones in which uPA activity was high. Thus, tPA seems to be the main PA involved in the proteolytic activity of whole SMC populations and clones in vitro (see Figure 3). Taken together, our results suggest that SMCs are heterogeneous in their proteolytic activities, at least as far as the PA system is concerned. Given what is known about the role of these proteases on extracellular matrix metabolism, our results suggest that epithelioid cells are more likely than spindle-shaped cells to be associated with migratory activity.

We have used SMC populations with distinct stable phenotypes, which can also be isolated by cloning. It has previously been reported that SMCs can modulate from a contractile to a synthetic phenotype (for review, see Reference 18). This approach has been very useful in understanding SMC phenotypic changes in models of experimental intimal thickening and possibly in human atheroma; however, it does not take into account the possibility of SMC heterogeneity.

uPA has traditionally been implicated in pericellular proteolysis during cell migration, is expressed in a wide range of tissues, and binds to a cell-surface receptor.\(^11\) In contrast, tPA is primarily involved in fibrin dissolution and is expressed by very few cell types. However, several studies have suggested that tPA and uPA both play a role in the formation of experimental intimal thickening in the rat.\(^19\)–\(^22\) Using the rat carotid model of arterial injury, Clowes et al\(^19\) demonstrated that uPA increases in the media at the onset of SMC proliferation, whereas tPA appears later, when SMCs begin to migrate from the media into the intima. This was confirmed by Jackson et al,\(^20\) who showed that tPA and uPA are upregulated during the first steps of intimal thickening: in particular, tPA is located in the superficial layers of the media, as is CRBP-1, one of the molecules that has been identified as a marker of the epithelioid phenotype.\(^15\) Using en face in situ hybridization and zymography, Reidy et al\(^13\) have shown that tPA and uPA as well as PAI-1 are expressed by SMCs migrating on the luminal surface of the injured rat aorta. uPA and uPAR mRNA are expressed during the first few days after balloon injury, whereas tPA is maintained at a high level even 6 weeks after endothelial injury. Therefore, it is not surprising that SMCs cultured as a whole population from the intimal thickening 15 days after endothelial injury and showing an epithelioid phenotype express mainly tPA and not uPA or uPAR. It is noteworthy that epithelioid clones, particularly those isolated from the normal media, exhibit the same features. This suggests that a subpopulation of medial SMCs, characterized by the capacity to acquire an epithelioid phenotype and high proteolytic activity in vitro, has the potential to develop features typical of the whole population derived from intimal thickening.
We have recently shown that epithelioid SMCs, cultured either as whole populations or as clones, display a higher migratory activity in vitro than do spindle-shaped SMCs. This correlates well with the increase in tPA activity observed in epithelioid SMCs. By use of several migration assays, it has also been demonstrated that tPA plays a key role in migratory activity of SMCs and endothelial cells. In addition, it has recently been discovered that human vascular SMCs express a high-affinity tPA receptor. Increased tPA activity is also relevant for the growth capacity of epithelioid SMCs in the absence of serum. Indeed, tPA has been suggested to be a potent mitogen for human aortic SMCs. These effects of tPA on migration and replication have been attributed to plasmin generation. Taken together, the present study and the studies cited above support a role for tPA in rat and human SMC extracellular proteolysis. Interestingly, uPA and its receptor appear to be essential for migration of human vascular SMCs. In addition, both uPA and uPAR have been detected in human atherosclerotic lesions of varying degrees of severity.

The development of mice lacking uPA, uPAR, tPA, PAI-1, and plasminogen (for review, see Reference 32) has clarified the role of these factors in experimental neointima development. Carmeliet and colleagues have demonstrated that in plasminogen-deficient mice, SMCs fail to migrate toward the intima, whereas their replication is unaffected. They have also demonstrated that neointima formation is reduced in uPA-deficient, uPA- plus tPA-deficient, but not tPA-deficient mice, suggesting that uPA is the major player in this process. In contrast, PAI-1–deficient mice develop a neointima more rapidly than do wild-type mice. Discrepancies observed between these results and ours could be explained by the different species used, as has been shown, for example, in mouse and rat ovarian granulosa cells.

PAI-1 activity was higher in spindle-shaped SMCs cultured as whole cell populations than in epithelioid SMCs. In contrast, PAI-1 mRNA expression was lower in spindle-shaped SMCs than in epithelioid SMCs. In addition, PAI-1 was complexed to tPA in all situations studied. Taken together, these results suggest that PAI-1 is sequestered by tPA in SMC populations derived from intimal thickening, which may explain the apparent reduction in its activity. In vivo, PAI-1 increases in the few hours after carotid endothelial injury, indicating that proteolytic activity depends on the balance between PA and PAI-1 expression.

We show in the present study that bFGF and PDGF-BB are able to significantly increase tPA activity in spindle-shaped and epithelioid cells, cultured as whole populations or as clones (summarized in the Table). However, both cytokines also increase PAI-1 activity. The observation that there is a net increase in plaque number and size in response to bFGF despite coinduction of tPA and PAI-1 points to the existence of a subtle mechanism regulating PA activity at the cell surface. bFGF and PDGF-BB play a key role in intimal thickening: in particular, they induce the migration of SMCs toward the intima and their subsequent proliferation. We show here that SMCs displaying the epithelioid phenotype exhibit a significant response to these factors by increasing tPA activity as well as tPA mRNA, whereas uPA is barely affected. In this regard, it is noteworthy that uPA is generally the PA stimulated by bFGF and PDGF-BB in endothelial cells as well as in SMCs isolated from species other than the rat. In related studies, Herbert et al have shown that PDGF-BB acts on SMC proliferation and migration via tPA, whereas bFGF acts through uPA.

TGF-β1 generally did not affect tPA and uPA activity in any SMC type, whereas it increased PAI-1 activity in all cases. Therefore, TGF-β1 would be expected to exert an antiproteolytic effect leading to low cell migratory activity. However, in vitro studies have shown that TGF-β1 increases migration of bovine SMCs and concomitantly decreases uPA and increases PAI-1. This implies the existence of complex mechanisms of regulation, including extracellular matrix components such as vitronectin. Surprisingly, only 2 spindle clones exhibited an increase in uPA activity in response to TGF-β1. It is noteworthy that the effects of TGF-β1 depend on several parameters, including the target cells. In particular, TGF-β1 has opposite effects on cell migration in SMCs and endothelial cells.

In conclusion, the present study demonstrates that rat arterial SMCs are heterogeneous with respect to their expression of the PAs. In particular, SMCs isolated from intimal thickening 15 days after endothelial injury exhibit a high proteolytic activity that is mainly due to high tPA activity and, to a lesser extent, low PAI-1. Strikingly, this feature is shared with cloned epithelioid cells even when they are derived from the normal media. This is compatible with the possibility that a subpopulation of medial SMCs characterized by an epithelioid phenotype is prone to migrate into the intima. The observation that bFGF and PDGF-BB enhance PA activity in epithelioid SMCs reinforces the possibility that PAs play an important role in the development of experimental intimal thickening.

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