Regulation of proteolytic enzymes and inhibitors in two smooth muscle cell phenotypes

Herbert K.F. Lau*

Department of Laboratory Medicine and Pathobiology, St. Michael’s Hospital and Department of Medicine, University of Toronto, 30 Bond Street, Toronto, Ontario, Canada M5B 1W8

Received 28 July 1998; accepted 18 March 1999

Abstract

Objective: Rat arterial smooth muscle cells (SMC) are diverse in nature. In addition to SMC cultures which grow to the typical ‘hill and valley’ morphology at confluence, there are other SMC which show spindle shape, or thin and long fusiform swirling pattern, or which have a cobblestone appearance at confluence. The growth of these cell types is variably dependent on serum growth factors, and they display different cytoskeletal proteins. We wish to study the secretion of proteolytic enzymes and enzyme inhibitors from these SMC which may be important for their biological activities. Methods: Two phenotypes, an ‘epithelioid-like’ SMC and a ‘swirling’ SMC, were isolated and cloned from rat carotid arteries. The proteolytic enzymes and inhibitors produced after stimulation with exogenous mediators were investigated with enzyme assays, zymography and immunoblotting. Results: Epithelioid SMC, but not swirling SMC, secreted MMP-2 in response to uP A and tP A. Epithelioid SMC produced small amounts of uP A and tP A in control cultures, but these proteinase secretions were enhanced by bFGF and PDGF. On the other hand, control swirling SMC secreted large amounts of uP A and tP A, which were reduced by the growth factors. In both cell types, the secretion of PAI-1 was stimulated by bFGF and PDGF, as well as by uP A and tP A. Furthermore, in both cell types, the secretion of TIMP-2 was enhanced by tP A and PDGF, but not by uP A or bFGF. Conclusions: When challenged with mediators, two rat SMC phenotypes behaved differently in terms of proteinase secretions, but they were similar in terms of proteinase inhibitor secretions. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Experimental; Regulatory systems; Biochemistry

1. Introduction

It is well recognized that SMC are heterogeneous in growth and functional properties [1–7]. The most common arterial SMC culture consists of spindle-shaped cells, which attain a ‘hill and valley’ morphology after reaching confluence, and their growth depends on growth factors provided by animal serum. A variant of this SMC show elongated, swirling, fusiform-shape cells at confluence [8]. Other SMC isolated from old rats, newborn rats or from the intimal thickening after balloon angioplasty, can grow independently of serum [9,10]. One such SMC, the π SMC phenotype [11], is similar to an ‘epithelioid-type’ SMC described [8]. They grow independently of serum, and to a cobblestone-shaped monolayer at confluence.

Plasmin is a proteolytic enzyme which directly breaks down extracellular matrix (ECM) [12,13], or indirectly through the activation of metalloproteinase (MMPs) zymogens [14,15]. The activated MMPs in turn digest ECM components such as collagen, laminin and proteoglycans. Two plasminogen activators, urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), cleave plasminogen to form plasmin. In vivo experiments have indicated that tPA and uPA increased after catheter injury in the rat [16], and tranexamic acid, a plasminogen activator inhibitor, can inhibit SMC migration after the injury [17]. These observations suggest a role for plasminogen activators in SMC functions. Furthermore, uPA appears to be more important than tPA, since uPA-knockout mice, as compared to wild-type or tPA-knockout mice, express a reduced rate and amount of neointima formation after carotid artery injury [18]. Increased MMP-2 and
MMP-9 expressions have also been observed following vascular denudation, and these proteinases are thought to be important for SMC function [19,20]. The proteolytic activities of plasminogen activators and MMPs are controlled by naturally-occurring inhibitors. An increase of the plasminogen activator inhibitor 1 (PAI-1), and the tissue inhibitor of matrix metalloproteinase 2 (TIMP-2) have been observed during recovery after balloon injury in the rat carotid artery [21]. This observation suggests that a proteolytic balance is essential for maintenance of proper functioning of the vascular wall.

We have isolated and cloned two SMC phenotypes from rat carotid arteries. We have measured the production of proteinases (uPA, tPA, MMP-2) and their inhibitors (PAI-1 and TIMP-2) in these cells, after stimulation with basic fibroblast growth factor (bFGF) or platelet-derived growth factor-BB (PDGF). We have also measured MMP-2 and the inhibitors after uPA and tPA stimulation. Our results show different proteolytic responses from the two cell types, which suggests that these cells may carry out different biological functions in the animal.

2. Methods

2.1. Materials

Recombinant human bFGF, PDGF-BB and single-chain tPA were obtained from Calbiochem (La Jolla, CA). Human plasminogen devoid of plasmin, and the Spectrolyse tPA assay were products of Biopool (Umeå, Sweden). High-molecular-weight uPA and antibodies against rat PAI-1 were from American Diagnostica (Greenwich, CT). Rabbit antibodies against human MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9 were obtained from Biogenesis (Poole, UK). Rabbit antibodies against TIMP-2 were obtained from BioScience International (Montreal, Quebec). Antibodies against von Willebrand factor, smooth muscle cell-specific α-actin and desmin were from Boehringer Mannheim (Laval, Quebec). Antibodies against smooth muscle myosin heavy chain were obtained from Biomedical Technologies (Stoughton, MA). Reagents used in cell culture were Gibco BRL products (Burlington, Ontario).

2.2. Cell culture

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996. Smooth muscle cells were obtained from the carotid arteries of Sprague Dawley rats using an explant method [22,23]. Briefly, after removing the adventitia, ~1 cm pieces of arteries were minced and cultured in DMEM containing 10 mmol/l non-essential amino acids, 100 u/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS), at 37°C in 95% air, 5% CO₂. Cells that migrated out of the arteries after 10–14 days were expanded. Confluent SMC were passaged using 0.05% trypsin and 0.2 mmol/l EDTA. To obtain specific SMC phenotypes, limited dilution cloning was carried out on a swirling SMC whole cell culture at the 5th passage, using 0.5–0.75 cell per well. Cloning cylinders were also used to isolate swirling and epithelioid cells. Small clusters consisting of ~10 cells which showed either swirling or epithelioid morphology were trypsinized from the 5th passage of a whole swirling SMC culture at ~70% confluence. Stimulation experiments were carried out in 100-mm culture plates, using confluent cells previously incubated in serum-free medium (DMEM containing non-essential amino acids, antibiotics and 0.5% bovine serum albumin (BSA)) for three days. On the day of the experiment, mediators were added to the cells in a total volume of 4 ml of fresh serum-free medium, and further incubated for 24 h at 37°C in 5% CO₂. Control cells received only 4 ml fresh serum-free medium. More than 95% of the control cells and cells which received mediators were viable at the end of the incubation. The cells were counted with a hemocytometer after incubation, and the conditioned media of duplicate culture plates were concentrated 4-fold with an Amicon YM-10 membrane, before being used for enzyme or inhibitor measurements.

2.3. Zymography

uPA activity was measured by zymography according to Heussen and Dowdle [24]. Briefly, concentrated conditioned media were normalized to equal cell numbers and electrophoresed in 10% SDS polyacrylamide gel containing 10 μg/ml plasminogen and 1% gelatin. In this system, uPA was more efficient than tPA in activating plasminogen. The lower limit of detection was approximately 0.1 unit/ml for uPA, while it took 10 unit/ml tPA to generate a comparable clear zone in the gel. 10 mmol/l EDTA was included in the buffer for developing PA activity in order to inhibit any MMP activity. Gelatinase activities were also measured using a modification of the above method. In this case, only gelatin and no plasminogen was premixed with acrylamide. The gelatinolytic enzymes directly lysed the gelatin to form clear zones in the Coomassie blue-stained background.

2.4. Western blot

After adjusting for equal cell numbers, concentrated conditioned media were electrophoresed in 11% SDS PAGE according to Laemmli [25], transferred onto nitrocellulose, and incubated with 2–5 μg/ml of primary antibodies for 18 h at 4°C. Horse radish peroxidase-linked secondary antibodies were then incubated with the nitrocellulose paper and proteins revealed with enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL).
2.5. **Immunofluorescence**

Indirect immunofluorescence was performed on methanol-permeabilized SMC grown on microscope slides as described [26]. SMC were reacted overnight with antibodies against von Willebrand factor, desmin, myosin heavy chain or smooth muscle specific α-actin. FITC-linked secondary antibodies (Becton Dickenson, Bedford, MA) were added and incubated for 1 h.

2.6. **tPA activity and antigen**

tPA activity was measured by a solid-phase fibrin assay [27]. tPA-like enzymes from SMC conditioned media were trapped on fibrin-coated wells, and their activity measured after addition of plasminogen and a plasmin substrate, val-leu-lys-p-nitroanilide (Bachem, Torrance, CA). The amount of tPA activity was compared to a standard curve set up with human single-chain tPA. tPA antigen was measured by the Spectrolyse tPA assay system of Biopool (Umea, Sweden) according to the manufacturer.

2.7. **uPA activity**

This was determined by an immunotrap assay of Kung and Lau [28]. After incubating conditioned media with wells that were previously coated with anti-human uPA antibodies, the trapped uPA-like activity was assayed by adding plasminogen and a plasmin chromogenic substrate, and the uPA activity was compared to a standard curve set up with human uPA. tPA and uPA activity were calculated as milliunit/ml/10^6 cells, and tPA antigen was calculated as ng/ml/10^6 cells. They are reported as percent of control cells, which received no mediator and were carried out in the same experiment.

2.8. **Statistics**

All experiments were conducted in two or three passages of three different epithelioid or swirling SMC clones. Data are presented as mean±SEM of the independent experiments. One-way analysis of variance (ANOVA) was performed using GraphPAD 1.13 (Intuitive Software, San Diego), in order to compare data between multiple groups. Tukey–Kramer test was performed for P<0.05 which was considered statistical significance.

3. **Results**

3.1. **Selection and characterization of epithelioid and swirling SMC cultures**

We have found that the most common SMC cultures from 6-month old rats consisted of either spindle-shaped SMC which grew to the ‘hills and valleys’, or thin, long cells which grew to whorls at confluence. We have selected cells with cloning cylinders and with limited dilution, using a whole SMC culture at the 5th passage. This culture consisted mainly of elongated cells which formed whorls with a fusiform pattern at confluence, but it also contained small patches of rounded, cuboidal-shaped (epithelioid-like) cells when observed at subconfluent density. Small patches of ~10 cells each of either swirling cells or epithelioid-typed cells were isolated with trypsin. Of 10 such selections, three were the parental swirling type, two showed epithelioid-shaped cells, and five grew into mixtures containing both types of cells. We selected one swirling and one epithelioid cultures, and followed them up to 9 additional passages. There were no morphological changes observed through these passages. Limited dilution cloning resulted in 24 cell clones, seven of which were propagated through 10 passages. Three swirling and three epithelioid-shaped cell lines were selected for further study. Whichever method was used to isolate the two types of SMC, the cell cultures with similar morphology behaved similarly in both their shape and secretion of proteinases and inhibitors, and therefore only the results derived from experiments of the cloned cells are described. Experiments involving incubation with mediators were carried out between passages 2 and 6.

The morphology of swirling and epithelioid SMC is shown in Fig. 1. In the presence of FBS, epithelioid cells grew slower than swirling cells. When plated at a density of 2×10^6 cells/well of 12-well plate, the former culture reached final numbers of approximately 70% of the latter after 6 days growth (average of three experiments). When deprived of serum, swirling SMC cultures stopped proliferating, whereas epithelioid cultures continued to grow, but at a rate only half as rapid as in the presence of 10% FBS.

None of the cell cultures reacted with von Willebrand factor antibodies. Whole SMC cultures and cloned cells reacted approximately 90% with SMC-specific α-actin antibodies. There was no discernable difference between whole SMC cultures and selected cell clones in their immunoactivity against myosin heavy chain, where 40–60% of the cells were positive. However, desmin was slightly higher in the swirling cells (20–30%) compared to epithelioid cells (~10%).

3.2. **MMP secretion**

Cultured SMC secreted a gelatinolytic activity of ~68 kDa which was usually associated with another protein band of slightly higher molecular weight. Both of these molecules reacted with antibodies against human MMP-2, suggesting that they were probably the respective active and inactive forms of MMP-2. On some zymographic analyses, a higher molecular weight MMP molecule of ~90 kDa, resembling human MMP-9 was also observed. The intensity of this band was much less than MMP-2. and Western blots performed on the rat SMC conditioned media using rabbit antibodies against human MMP-9 failed.
to show any crossreactive protein. The small amount of MMP-9 found in these cells relative to MMP-2 may have resulted from less effective gelatinolytic activity of MMP-9 and/or less immuno-crossreactivity with the anti-human protein antibodies. Furthermore, there was no detectable variation of this MMP molecule in zymography after incubation with any of the mediators used. Antibodies against human MMP-1, MMP-3 and MMP-7 also failed to reveal any crossreactive proteins in Western blots (data not shown).

For epithelioid SMC, a concentration-dependent increase of MMP-2 gelatinolytic activity and immuno-reactivity was observed when uPA (Fig. 2A,B), or tPA (Fig. 2E,F) was used to stimulate these cells. For swirling SMC, the secreted MMP-2 enzyme activity and immuno-reactivity from the conditioned media remained the same as control cells, after stimulation by uPA (Fig. 2C,D), or tPA (Fig. 2G,H). These figures represent individual experiments, and similar results from three clones of each cell type have been observed. bFGF and PDGF had no effect on MMP-2 secretion from either cell type (data not shown).
3.3. uPA secretion

The basal concentration of secreted uPA from three epithelioid SMC clones was 6.2±2.2 milliunit/ml/10^6 cells. bFGF or PDGF stimulation caused a concentration-dependent increase in uPA that was most pronounced around 0.1–1 ng/ml of each stimulator. Fig. 3A and B show typical zymographs of uPA secretion induced by bFGF and PDGF, respectively. Fig. 3C shows uPA activity measurements using a plasmin substrate, obtained from data of three individual epithelioid cell clones. There was a general agreement between these two assays. However, the signal in the zymography appears to be not linearly related to the activity measurements.

The basal concentration of uPA secreted by three different clones of swirling SMC was 231±86 (mean±SEM) milliunit/ml/10^6 cells (n=4), which was ~35 fold more than that of control epithelioid SMC. The uPA secretion from the swirling SMC was reduced relative to the control cells, after incubation with bFGF (Fig. 4A) or PDGF (Fig. 4B). Fig. 4C shows the uPA secretion as measured by a plasmin chromogenic substrate, which, in general, agrees with zymography.

3.4. tPA secretion

For epithelioid SMC, tPA activity of the control cells was 102±17 milliunits/ml/10^6 cells, and that of tPA antigen concentration was 66±14 ng/ml/10^6 cells. Most bFGF concentrations stimulated more tPA than control cells. The changes in tPA generally followed a biphasic response, similar to that of uPA secretion. Fig. 5A shows that bFGF-induced tPA secretion appears to plateau between 0.1 and 1 ng/ml bFGF. Fig. 5B shows that tPA secretion in response to PDGF was similar but smaller than that due to bFGF. It also shows that tPA antigen changes (open bar) are not significantly different (ANOVA P=0.93), although they follow a similar trend as tPA activity (closed bar).

For swirling SMC, tPA activity of the control cells was 558±77 milliunits/ml/10^6 cells (n=6), and that of tPA antigen concentration was 87±19 ng/ml/10^6 cells (n=6). Control cells which received no stimulus secreted more tPA than cells incubated with bFGF or PDGF (Fig. 5C,D). Fig. 5C,D show, respectively, that tPA activity (closed bars) follows a biphasic response to bFGF and PDGF. The tPA antigen concentrations (open bars) induced by the
Fig. 3. Secretion of uPA in epithelioid SMC by bFGF and PDGF (A,B). Zymography was performed in the presence of plasminogen (see Methods for details), and was used to assay uPA activity of the conditioned media of epithelioid SMC that had been incubated with various concentrations of bFGF (A) or PDGF (B). The control cells that had not received mediator is labelled as C in the figure. Similar results have been obtained from three epithelioid clones. (C) The uPA activities of the conditioned media of epithelioid SMC as measured by a plasmin chromogenic substrate (see Methods for details) are expressed as percentages of the control cells. The uPA activity of control cells from three epithelioid clones was 6.2±2.2 (mean±SEM) milliunits/ml/10^6 cells (n=6). Shaded bars represent uPA induced by bFGF, and open bars represent uPA induced by PDGF.

growth factors are not significantly different from each other (ANOVA P=0.21 and 0.28 respectively), although they appear to follow the trend of tPA activity measurements. The reason(s) for the discrepancy between the tPA activity and antigen measurements was not clear. The antigen measurement made use of anti-human tPA antibodies, which may have reduced crossreactivity with the rat molecule, or they may not be able to distinguish free tPA and tPA complexed with PAI-1, which is the most likely form of the enzyme found in the conditioned medium.

3.5. PAI secretion

PAI-1 was up-regulated by uPA, tPA, bFGF and PDGF in a concentration-dependent manner, in both epithelioid and swirling SMC. Fig. 6 shows representative Western blots results, obtained from four independent experiments using three different epithelioid and swirling SMC clones. Fig. 6 A, C, E, G show Western blots against rat PAI-1 antibodies from the conditioned media of epithelioid SMC, after stimulation with uPA, tPA, bFGF and PDGF respectively. Fig. 6 B, D, F, H show the corresponding responses from swirling SMC.

3.6. TIMP-2 secretion

TIMP-2 secretion into the conditioned media was enhanced by bFGF or tPA in a concentration-dependent manner, in both epithelioid (Fig. 7A,C) and swirling SMC (Fig. 7B,D). uPA and PDGF had no effect on the secretion of TIMP-2 in either cell type (data not shown). The figures are representative of four experiments using three different epithelioid and swirling SMC clones.
4. Discussion

We have isolated and cloned two types of smooth muscle cells from normal rat arterial media, which display different physical and proteolytic properties. SMC phenotypes have been described in SMC cultures of rats of different ages [11,29–32]. Whole SMC cultures containing predominantly spindle-shaped or epithelioid cell types have been described [2,10], and cloned epithelioid SMC have been produced [8,33,11]. When compared to our cell lines, there are some differences to how these various epithelioid cells were produced. Our cell lines were derived from carotid arterial media using an explant method, whereas thoracic aortic media digested with collagenase were the origins of the epithelioid-type SMC reported previously. We used a 5th passaged whole SMC culture for cloning experiments while one group used a 14th-passaged culture [11,33], and another group used primary cultures from normal media and from intimal thickening 15 days after balloon injury for their cloning [8]. Despite these differences, our epithelioid cell clones appeared to be generally similar in morphology, growth and cytoskeletal characteristics to these previously reported cells. It is interesting to note that an epithelioid-type SMC
was found in 18-month old rats, which was similar to our epithelioid cell lines in that they were able to continue to grow in the presence of 10% FBS after reaching confluence [29]. Our swirling-type of SMC clones also appeared similar to one of the three SMC phenotypes, in addition to the epithelioid cell type of Bochaton-Piallat et al. [8]. These cell culture results would suggest that there are only a limited number of sub-populations of SMC, which can be isolated from different blood vessels.

In this study we have tried to show that these phenotypes are different in their profile of secreted proteinases and inhibitors when stimulated by various mediators. Our results show that the epithelioid-type SMC can be induced to secrete MMP-2 by uPA and tPA. Even though the basal levels of uPA and tPA in epithelioid SMC were low, they could be enhanced by most concentrations of bFGF and PDGF. In the presence of growth factors, the epithelioid SMC may first produce plasminogen activators, which in turn may induce MMP-2, and these cells thus attain proteolytic activity to degrade surrounding proteins. This may suggest that the epithelioid cell type is more proteolytic in an environment where growth factors are found. This could occur, for instance, during the early phase of repairing a damaged blood vessel where small number of platelets and macrophages start to gather at the site of the wound. However, in the same cells, secretion of
Fig. 6. Induction of PAI-1 by uPA, tPA, bFGF and PDGF Confluent epithelioid or swirling SMC were incubated with serum-free medium containing non-essential amino acids, antibiotics and 5% BSA for three days. Afterwards, 4 ml of fresh serum-free medium containing various concentrations of uPA, tPA, bFGF or PDGF were added to the cells, and further incubated for 24 h. The conditioned media were collected from duplicate plates of cells, concentrated four-fold, and used for immunoblot experiments, after adjusting for cell numbers. The sample of control cell conditioned media without addition of mediator is denoted as ‘C’ in the figure. Panels A, C, E, G show Western blots against rat PAI-1 antibodies from the conditioned media of epithelioid SMC, after stimulation with uPA, tPA, bFGF and PDGF respectively. Panels B, D, F, H show the corresponding responses from swirling SMC.

Fig. 7. Induction of TIMP-2 by tPA and bFGF Confluent epithelioid and swirling SMC were treated with bFGF and tPA, and the conditioned media were processed for Western blots against TIMP-2 antibodies as described before. Panels A and C are from representative epithelioid cells and panels B and D are from representative swirling cells.
the protease inhibitor PAI-1 can be induced by uPA, tPA, bFGF and PDGF, while TIMP-2 can be induced by tPA and PDGF. Therefore, the final proteolytic equilibrium of epithelioid SMC will depend on the magnitude and timing of producing these pro- and anti-proteolytic molecules.

In contrast, the swirling SMC secrete high levels of uPA and tPA in control cells, which can be down-regulated by bFGF and PDGF. These cells do not produce MMP-2 in response to growth factor stimulation. Furthermore, bFGF, PDGF, uPA and tPA all induce secretion of PAI-1, while tPA and bFGF induce TIMP-2 secretion. Therefore in the presence of growth factors, swirling SMC will always evoke an anti-proteolytic potential.

Taken together, our results show that growth factors in the microenvironment exert different effects on these two SMC phenotypes. In order to understand the true functions of these SMC phenotypes, one has to know precisely when and where these different cell types arise, and how much of regulatory growth factors and chemokinones surround them. Without in vivo experiments to further analyze these factors, our present study can only provide speculative bases of the purported importance of their secreted proteinase and inhibitors. During the submission of this manuscript, a paper describing rat aortic SMC phenotypes has appeared [34]. Our data appear to confirm similar conclusions that SMC are functionally heterogeneous with respect to the profile of secreted proteinases and inhibitors, which is highly regulated by growth factors.

Acknowledgements

This study was supported by the St. Michael’s Hospital Research Society. The author wishes to thank Dr. Jerry Teitel for his encouragement and critical review of the manuscript.

References


