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Vascular Smooth Muscle Cells Efficiently Activate a New Proteinase Cascade Involving Plasminogen and Fibronectin

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Abstract The plasminogen/plasmin system is involved in vascular wall remodeling after injury, through extracellular matrix (ECM) degradation and proteinase activation. Vascular smooth muscle cells (VSMCs) synthesize various components of the plasminogen/plasmin system. We investigated the conversion of plasminogen into plasmin in primary cultured rat VSMCs. VSMCs efficiently converted exogenous plasminogen into plasmin in a time- and dose-dependent manner. We measured plasmin activity by monitoring the hydrolysis of Tosyl-G-P-R-Mca, a fluorogenic substrate of plasmin. Cell-mediated plasmin activation was associated with the degradation of ECM, as revealed by fibronectin proteolysis. Plasmin also activated a proteinase able to hydrolyze Mca-P-L-G-L-Dpa-A-R-NH_2, a fluorogenic substrate of matrix metalloproteinases (MMPs). However, this proteinase was not inhibited by an MMP inhibitor. Furthermore, this proteinase displayed similar biochemical and pharmacological properties to fibronectin-proteinase, a recently identified zinc-dependent metalloproteinase located in the gelatin-binding domain of fibronectin. These results show that VSMCs convert exogenous plasminogen into plasmin in their pericellular environment. By hydrolyzing matrix protein plasmin activates a latent metalloproteinase that differs from MMP, fibronectin-proteinase. This metalloproteinase may participate to vascular wall remodeling, in concert with other proteinases. J. Cell. Biochem. 88: 1188–1201, 2003.

Key words: vascular smooth muscle cells; plasminogen; fibronectin; Fn-proteinase

The plasminogen/plasmin system is involved in vessel wall remodeling after injury [for review, see Carmeliet and Collen, 1998]. This system consists of an inactive proenzyme, plasminogen, which is converted to the serine protease plasmin by an urokinase-type plasminogen activator (uPA) and by tissue-type plasminogen activator (tPA). This cascade is tightly regulated at two levels. First, the activity of the plasminogen activators (PAs) is controlled by plasminogen activator inhibitors (PAIs). PAI-1 appears to be the main physiological inhibitor. Plasmin activity can be inhibited by α_2-antiplasmin. Plasmin may be involved in the control of cell proliferation [Herbert et al., 1997], apoptosis [Herbert and Carmeliet, 1997], and migration [Herbert et al., 1997] during the remodeling of vessel walls. In addition, plasmin may affect the organization of the ECM through the proteolysis of some ECM components like fibronectin (Fn) and laminin, and through the activation of matrix

Abbreviations used: APMA, *p*-aminophenylmercuric acetate; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; ECM, extracellular matrix; Fn, fibronectin; GBD, gelatin-binding domain; Mca, (7-methylcoumarin-4-yl)acetyl; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; SDS/PAGE, sodium dodecylsulfate/polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TIMP, tissue inhibitor of matrix metalloproteinase; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; VSMCs, vascular smooth muscle cells; Z, benzyloxycarbonyl.

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metalloproteinases (MMPs) [He et al., 1989; Carmeliet et al., 1997].

MMPs constitute a growing family of zinc-dependent metalloproteinases able to degrade virtually all ECM components. MMPs are synthesized as latent pro-enzymes, which are activated by limited proteolysis. This activation step is critical for the regulation of MMP activity. In addition, MMP activity is controlled by tissue-inhibitors of metalloproteinases (TIMPs). MMPs are involved in vascular remodeling associated with vascular diseases and injury [Allaire et al., 1998; George et al., 1998; Moore et al., 1999; Pyo et al., 2000].

Functional interactions have been described between plasminogen/plasmin and MMP systems, suggesting that both systems cooperate in tissue remodeling processes. MMPs can be activated by plasmin [He et al., 1989; Carmeliet et al., 1997]. Moreover, several MMPs are able to generate the angiogenesis inhibitor, angiostatin, from plasminogen [Patterson and Sang, 1997; Lijnen et al., 1998; O'Reilly et al., 1999] and MMP-3 can inactivate PAI-1 [Lijnen et al., 2000].

VSMCs contribute prominently to vascular structure and function through the synthesis and organization of the ECM and through the regulation of vascular tone. VSMCs synthesize various components of the plasminogen/plasmin system and are a source of MMPs in the vascular wall. The expression of PAs and MMPs is significantly induced after injury [Clowes et al., 1990; Galis et al., 1994b; Thompson et al., 1995; Reidy et al., 1996]. During physiopathological remodeling processes, VSMCs may therefore control their pericellular environment by activating plasmin, which in turn activates MMPs. As PAs and MMPs are synthesized by cell types other than VSMCs in the remodeling vascular wall, it is difficult to study this process in vivo. In this study, we investigated the conversion of exogenous plasminogen into plasmin in primary cultured rat VSMCs. Our results indicated that VSMCs efficiently activate plasmin in a cell-dependent manner. Plasmin activation, in turn, induces ECM degradation, as demonstrated by Fn proteolysis.

Furthermore, our results showed that the generation of plasmin activates of a proteinase that can hydrolyze Mca-P-L-G-L-Dpa-A-R-NH_2, a fluorogenic substrate of collagenase. This fluorogenic peptide is a substrate for MMPs [Knight et al., 1992] and for a recently described cryptic zinc-dependent metalloproteinase, located in the gelatin-binding domain (GBD) of Fn [Boudjennah et al., 1998; Schnepel and Tschesche, 2000]. This proteinase, called Fn-proteinase, degrades denatured collagen I, laminin and collagen IV [Lambert Vidmar et al., 1991] and may be involved in cartilage matrix turnover [Chevalier et al., 1996].

Our results indicate that VSMCs activate plasmin, which affects the pericellular environment of VSMCs by degrading the ECM and activating a proteinase.

MATERIALS AND METHODS

Materials

A rabbit polyclonal antibody directed against rat plasma fibronectin was purchased from Biogenesis (Poole, England, UK). Mca-P-L-G-L-Dpa-A-R-NH_2 was from Bachem Biochimie (France). Plasmin, plasminogen, pefabloc® and MMP-2 were purchased from Roche Diagnostics (France). Bovine fibronectin, *p*-aminophenylmercuric acetate (APMA), α_2-antiplasmin, skin bovine gelatin, bovine milk α-casein, and Tosyl-G-P-R-Mca were from Sigma Saint Quentin Fallavier, France. A monoclonal anti HA-epitope antibody (clone 12CA5) was purchased from Roche and monoclonal antibody IST 10 was purchased from Chemicon. Batimastat was a gift from British Biotechnologies (UK).

Cell Culture

Normotensive male Wistar rats (160–180 g) were obtained from Iffa Credo (France). They were handled in accordance with the European Community standards concerning the care and use of laboratory animals (Ministère de l'Agriculture, France; authorization no. 00577).

VSMCs were isolated from rat thoracic aorta by enzymatic dissociation as previously described [Battle et al., 1994]. VSMCs were grown in DMEM (GIBCO, Cergy-Pontoise France) supplemented with 10% fetal calf serum (GIBCO), 100 U/ml penicillin (GIBCO), and 100 U/ml streptomycin (GIBCO), in a 5% CO_2/95% air humidified atmosphere at 37°C. The growth medium was changed every 2 or 3 days. Passages 3–18 were used for experiments.

Cells were plated at 40,000 cells/well in six-well-culture plates. After 6 days growth, cells were rinsed twice with phosphate buffered saline, and left in serum-free medium for 48 h. After two more washes with phosphate buffered

saline, fresh serum-free medium with or without plasminogen was added to the cultures. After various incubation times, the conditioned media were sampled, centrifuged for 5 min at 5,000 *g* and then concentrated 10-fold by centrifugation (ultrafree-4 biomax-10 kDa membrane, Millipore). Concentrated conditioned media were stored at −20°C until use.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from VSMCs by use of the RNeasy® mini Kit (Qiagen), according to the manufacturer's instructions. Total RNA (4 μg) was subjected to reverse transcription using Supersript II RT (Gibco), according to the manufacturer's instructions.

For each PCR, 0.5 μl cDNA was mixed with 10 pmol of sense and antisense primers, 0.2 mM dNTPs, 3 μl 10 × Taq polymerase buffer and 1 U Taq polymerase (Roche), in a final volume of 30 μl. A 31-cycle PCR reaction was used. Each cycle consisted of 45 sec at 94°C, 45 sec at 58°C, and 1 min at 72°C. The reaction mixture (15 μl) was then subjected to electrophoresis in 1.0% agarose gel in 0.5 × TAE (20 mM Tris-acetate and 0.5 mM EDTA) buffer. DNA was visualized by ethidium bromide staining.

The following primers were used:

- rat tPA sense 5′-CCATGGAATTCCATGATCCTG-3′ and antisense 5′-GACTAGTCATTGCTTCATGTTGTCTTG-3′;
- rat uPA sense 5′-TCCTGACCAGGATGGAAATC-3′ and antisense 5′-AGTGGAGCATCACGGAAGAC-3′;
- rat PAI-1 sense 5′-CCTCCACACACAAAACATGG-3′ and antisense 5′-CTCTGCCTCCCAAGTGCTAC-3′;
- rat plasminogen sense 5′-TCTGGACAGCACTTCTGTGG-3′ and antisense 5′-CCAATTAACGTACCGGGAAA-3′;
- rat MMP-2 sense 5′-AGCGTGAAGTTTGGAAGCAT-3′ and antisense 5′-ATGTCAGACAACCCGAGTCC-3′;
- rat MMP-9 sense 5′-ACGCTGTCCTTTCTTGTTGG-3′ and antisense 5′-CCTGTGAGTGGGTTGGATTC-3′.

The expected sizes of the PCR products were 981, 498, 498, 603, 603, and 592 bp for tPA, uPA, PAI-1, plasminogen, MMP-2 and MMP-9, respectively.

Enzymatic Assays

Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis. Aliquots of 10-fold concentrated conditioned media were incubated in 100 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM $CaCl_2$, with 44 μM of the intramolecularly quenched fluorogenic substrate of collagenase, Mca-P-L-G-L-Dpa-A-R-NH_2 [Knight et al., 1992], for 24 h at 37°C. Fluorescence was measured in a Kontron SFM 25 spectrofluorometer at 326 nm (excitation) and 395 nm (emission). The Mca-P-L peptide was used for calibration as previously described [Boudjennah et al., 1998].

The hydrolysis of Mca-P-L-G-L-Dpa-A-R-NH_2 by conditioned media of plasminogen treated cells was determined in the same conditions as described above. Samples were incubated with inhibitors for 10 min at room temperature prior to the addition of the substrate.

The concentrations of APMA, a proMMP activator, plasmin, inhibitors and antibodies used are indicated in the figure legends.

Tosyl-G-P-R-Mca hydrolysis. Reactions were performed as described above in 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0,01% Tween-20, and 62 μM Tosyl-G-P-R-Mca, a plasmin substrate [Lottenberg et al., 1981], for 2 h at 37°C. Fluorescence was measured at 347 nm (excitation) and 440 nm (emission).

Processing of Fibronectin by Plasmin

Bovine fibronectin (10 μg) was incubated with plasmin (6 μg) in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$ for 4 h at 37°C. After processing, Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis was determined. Samples were incubated with inhibitors for 10 min at room temperature before adding the substrate (44 μM). Reactions were allowed to proceed for 2 h at 37°C before measuring fluorescence, as described above.

For time- and dose-dependency studies, the same procedures were used in presence of various amounts of plasmin (0–6 μg) and fibronectin (3.3–10.0 μg).

Zymography

Samples were loaded onto 10% SDS/PAGE gels containing bovine skin gelatin or α-casein from bovine milk (1.2 mg/ml). After electrophoresis, SDS was removed by two 1 h washes in 12.5 mM Tris/HCl, pH 7.4, 2.5% Triton X-100, followed by one 30-min wash in a buffer

containing 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$ and incubated over-night at 37°C in the same buffer. Lysis bands were revealed by Coomassie brilliant blue R-250 staining.

Immunoblotting

After being subjected to 10% SDS–PAGE, samples were transferred onto PVDF membranes (Immobilon P, Millipore). The membrane was probed with antibodies directed against rat plasma Fn (dilution 1:1,000) and bound antibodies were revealed with the biotin-streptavidin system [Wei et al., 1991].

RESULTS

VSMCs Are Able to Convert Plasminogen Into Plasmin

We tested for the presence of various components of the plasminogen/plasmin cascade in VSMCs. RT-PCR with specific oligonucleotides revealed that tPA, uPA, and PAI-1 mRNAs were present in VSMCs and no RT-PCR product was detected for plasminogen in the same conditions (Fig. 1A) whereas plasminogen mRNA was observed in rat liver (data not shown). The presence of PAs and PAIs in VSMCs suggests that these cells convert exogenous plasminogen into plasmin in their pericellular environment. We then cultured VSMCs in the presence of exogenous plasminogen and analyzed the conditioned media for plasmin activity by use of Tosyl-G-P-R-Mca, a plasmin-fluorogenic substrate, and casein zymography.

Conditioned media from control cells, cultured in the absence of exogenous plasminogen, did not hydrolyze Tosyl-G-P-R-Mca (Fig. 1B, lane 1). In addition, caseinolytic activity was not observed in zymography (Fig. 1C, lane 1). These results suggest that no plasmin-like activity is present in conditioned media of VSMCs. The treatment of VSMCs with exogenous plasminogen led to the strong hydrolysis of the plasmin substrate (Fig. 1B, lane 2). This hydrolysis was completely inhibited when α_2-antiplasmin, a natural inhibitor of plasmin, was added (Fig. 1B, lane 3). In addition, treatment of VSMCs with exogenous plasminogen generated of 93 and 41 kDa bands, typical of caseinolytic activities (Fig. 1C, lane 2). The 93 kDa band corresponded to plasminogen. A similar band was observed when plasminogen was loaded as a control (Fig. 1C, lane 5). Plasminogen probably activated itself in the gel, as it did not display any Tosyl-G-P-R-Mca hydrolyzing activity (Fig. 1B, lane 4). The 41 kDa protein cross-reacted with an antiserum raised against plasminogen (data not shown) and formed a stable complex with α_2-antiplasmin (Fig. 1C, lane 3), suggesting that it is a catalytically active plasmin fragment. To prevent the automatic conversion of plasminogen into plasmin, plasminogen was incubated in cell-free medium. Neither Tosyl-G-P-R-Mca hydrolysis nor the 41 kDa band was detected (Fig. 1B,C, lane 4). These results indicate that the conversion of plasminogen into plasmin is cell-mediated. Furthermore, plasmin activity was first detected 16 h after plasminogen treatment (7.5 μg/ml) and then increased in a time-dependent manner (Fig. 2A). Plasmin activation occurred mainly during the first 24 h of treatment and slowed down between 24 and 48 h (Fig. 2A). Plasmin activation was also dose-dependent (Fig. 2B). Plasmin activity was observed upon the addition of 1.5 μg/ml plasminogen and increased linearly up to 15 μg/ml of plasminogen. Higher concentrations of plasminogen were not tested as concentrations of above 15 μg/ml induced cell death. The time- and dose-dependent increase of Tosyl-G-P-R-Mca hydrolysis observed upon plasminogen treatment was correlated with the appearance of the 41 kDa band (Fig. 2A,B, inset). These results show that VSMCs efficiently convert plasminogen into plasmin.

In solution, the activation of plasmin may occur in a soluble PAs-dependent manner. Alternatively, PAs may activate plasmin at the surface of VSMCs. To address this question, VSMCs were cultured in the presence and absence of plasminogen (7.5 μg/ml) for 48 h. Conditioned media were then harvested and media from VSMCs cultured in the absence of plasminogen were incubated with and without plasminogen (7.5 μg/ml) for a further 24 h. Tosyl-G-P-R-Mca hydrolysis was not detected in conditioned media from VSMCs cultured in the absence of plasminogen or in the same media incubated without plasminogen for 24 additional hours (data not shown). Considerably more hydrolysis was observed in conditioned media from plasminogen-treated cells than in to those from VSMCs cultured in the absence of plasminogen and then incubated for an additional 24 h with plasminogen (128.3 ± 5.4 and 15.2 ± 2.1 fluorescence arbitrary units,

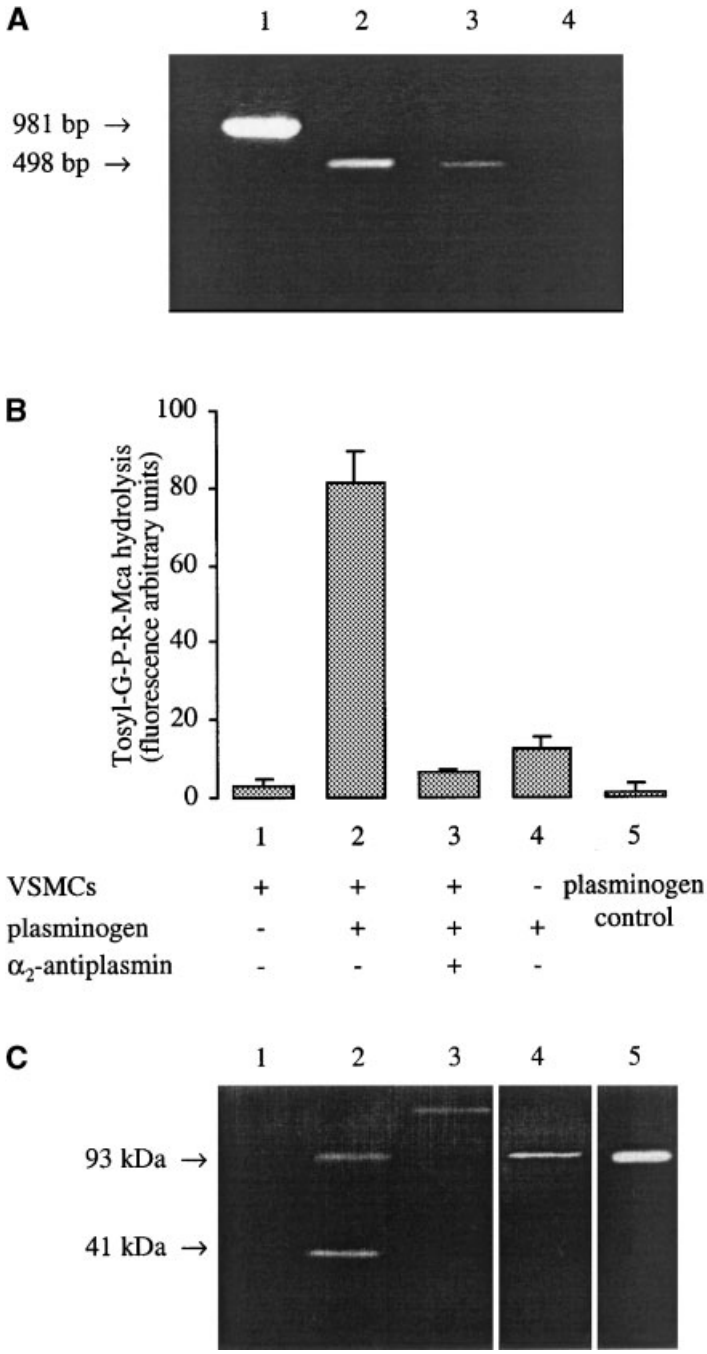

Fig. 1. VSMCs convert exogenous plasminogen into plasmin. **A**: cDNAs obtained from VSMCs were used as templates for PCR with specific primers for rat tPA (**lane 1**), uPA (**lane 2**), PAI-1 (**lane 3**), and plasminogen (**lane 4**). **B**, **C**: VSMCs were incubated in serum-free DMEM with or without plasminogen (7.5 μg/ml) and α_2-antiplasmin (5 μg/ml) for 48 h. Conditioned media were then collected and assayed for Tosyl-G-P-R-Mca hydrolysis (B). Data are presented as the mean ± SEM for three experiments performed in duplicate. C: Casein zymography of conditioned media. **Lanes 1–3** represent the activity of conditioned media from untreated VSMCs (lane 1); from plasminogen (7.5 μg/ml) treated VSMCs (lane 2); and from VSMCs treated with plasminogen (7.5 μg/ml) and α_2-antiplasmin (5 μg/ml). **Lanes 4** and **5** represent the activity of plasminogen (7.5 μg/ml) incubated in serum-free DMEM without cells and of pure plasminogen (1.5 μg), respectively.

respectively, n = 6). These results show that plasmin is only efficiently activated in the presence of VSMCs and suggest that this mechanism takes place at the cell surface.

Plasmin Activation Induces Proteolysis of VSMCs Derived Fibronectin

The proteolysis of the ECM is one of the many functions of plasmin activation by VSMCs. To assess this function, Fn proteolysis was analyzed. Fn was detected in conditioned media after 16 h of culture as a 220 kDa species, corresponding to the native Fn monomer (Fig. 3A). No Fn proteolysis was observed even after 48 h in culture, despite the clear accumulation of Fn (Fig. 3A). In contrast, Fn proteolysis was observed in conditioned media from plasminogen-treated cells (Figs. 3B and 4). Fn fragments of 96, 56, and 42 kDa appeared in the conditioned media after 16 h of treatment

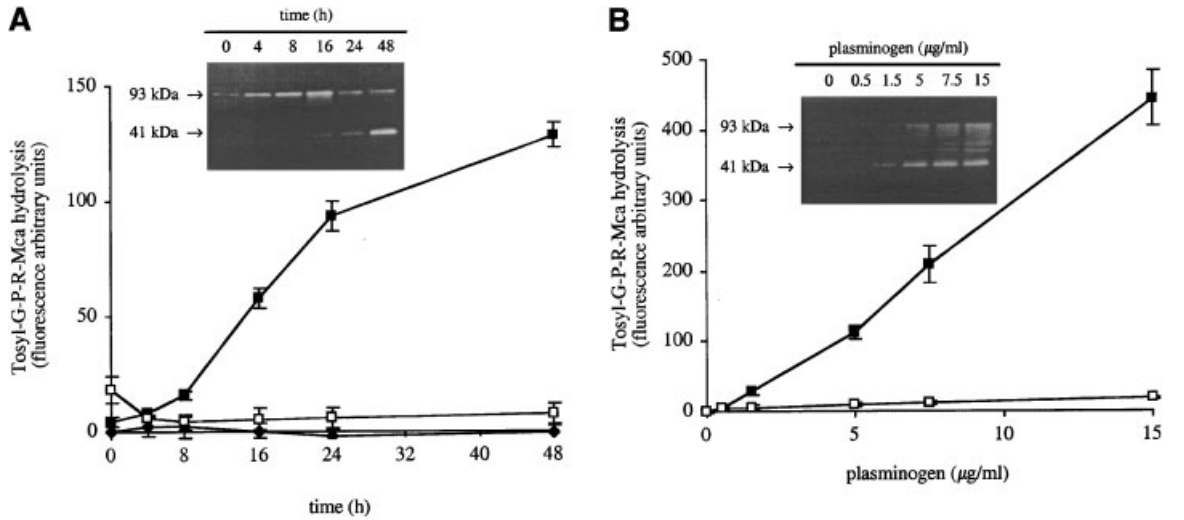

Fig. 2. Time- and dose-dependent activation of plasmin by VSMCs. **A**: Time-course of plasmin activation. VSMCs were incubated in serum-free DMEM with (■) or without plasminogen (7.5 μg/ml) (◆) for the indicated times. As a control, plasminogen (7.5 μg/ml) was incubated in serum-free DMEM without cells (□). **B**: Dose-dependent activation of plasmin. Plasminogen (0–15 μg/ml) was incubated in serum-free DMEM with (■) or without VSMCs (□) for 48 h. At the end of experiment, conditioned media were collected and analyzed for plasmin activity by measuring Tosyl-G-P-R-Mca hydrolysis. The data presented are the mean ± SEM for three experiments performed in duplicate. **Inset**: the plasmin activity of conditioned media from plasminogen-treated cells harvested in the same experiments was analyzed by casein zymography.

(7.5 μg/ml plasminogen) or after the addition of 0.5 μg/ml of plasminogen for 48 h (Figs. 3B and 4). The Fn proteolysis kinetics and dose-dependency mirrored the cellular plasmin activation reported in Figure 2A,B. These results suggest that VSMC-mediated plasmin activation modifies the cell environment as a result of ECM proteolysis.

VSMC-Mediated Plasmin Activation Activates Proteinase

RT-PCR showed that VSMCs contained MMP-2 mRNA (Fig. 5A). In conditioned media, MMP-2 was also detected by gelatin zymography (Fig. 5B) and immunoblotting (not shown). After 16 h in culture, MMP-2 appeared as a doublet of 73 and 67 kDa, corresponding to inactive forms of the enzyme (Fig. 5B). In contrast, MMP-9 messenger was not detected by RT-PCR on VSMCs (Fig. 5A) whereas it was observed in rat lung (data not shown). In addition, MMP-9 protein was not detected by gelatin zymography (Fig. 5B). Conditioned media induced a weak hydrolysis of Mca-P-L-G-L-Dpa-A-R-NH_2, a collagenase fluorogenic substrate [Knight et al., 1992]. This hydrolysis was not inhibited by batimastat, an MMPs inhibitor (Fig. 5C). Treatment of conditioned media with APMA, a specific MMP activator, induced the appearance of the 62 kDa active form of MMP-2 and strong hydrolysis that was totally inhibited by batimastat (Fig. 5B,C). These results suggest that VSMCs produce an inactive precursor of MMP-2.

Another candidate for proteinase activity is the GBD of Fn. The GBD of Fn displays a zinc-dependent collagenase activity and is able to hydrolyze Mca-P-L-G-L-Dpa-A-R-NH_2 [Boudjennah et al., 1998]. This collagenase, called Fn-proteinase, is inhibited by a phosphinic peptide inhibitor, Z-FΨ(PO_2CH_2)A-R-F-OH, and is not affected by MMP inhibitors [Boudjennah et al., 1998]. This peptide was used to detect Fn-proteinase activity in conditioned media. No inhibition was obtained using Z-FΨ(PO_2CH_2)A-R-F-OH (Figure 5C), showing that VSMCs do not constitutively produce active Fn-proteinase.

Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis increased after treatment with plasminogen (175.9 ± 9.2 nM of Mca-P-L liberated by the control vs 474.8 ± 63.2 nM by plasminogen treated cells, n = 8). Plasmin did not hydrolyze this substrate (data not shown). Nevertheless, this increased hydrolysis was dependent on plasmin enzymatic activity, as it was strongly inhibited (80% inhibition) by α_2-antiplasmin (231.2 ± 15.0 nM of Mca-P-L liberated, n = 8).

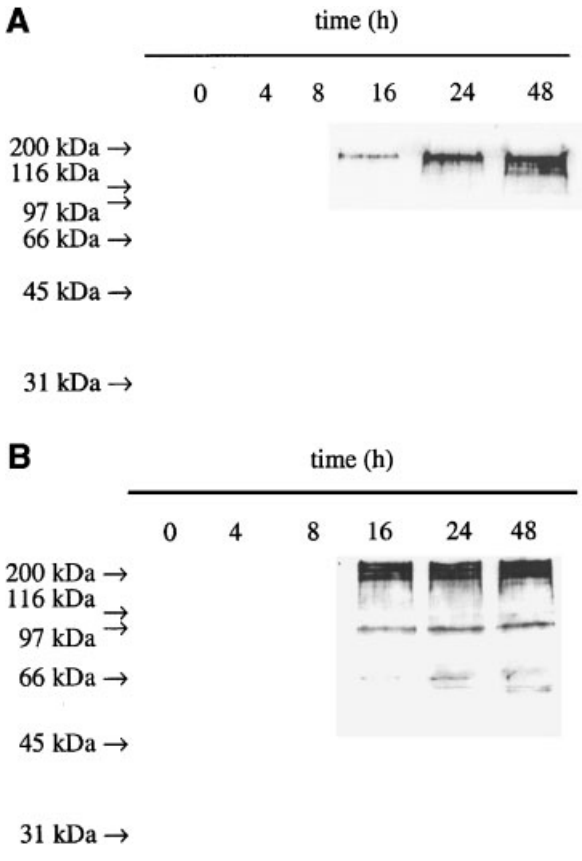

Fig. 3. VSMCs-mediated plasmin activation induces fibronectin proteolysis. VSMCs were incubated in serum-free DMEM in the absence (**A**) or presence of plasminogen (7.5 μg/ml) (**B**) for the indicated times. Conditioned media (8 μl of 10-fold concentrated media) were then analyzed by immunoblotting.

Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis increased in a time-dependent manner after plasminogen treatment; starting after 16 h (2.8-fold increase) and reaching a plateau after 24 h (5.8-fold increase) (Fig. 6A). Substrate hydrolysis induced by plasminogen was dose-dependent; it was detectable at a concentration of 1.5 μg/ml and increased linearly to 15 μg/ml (Fig. 6B). These results strongly suggest that the plasmin produced in VSMCs activates an enzyme responsible for the hydrolysis of Mca-P-L-G-L-Dpa-A-R-NH_2.

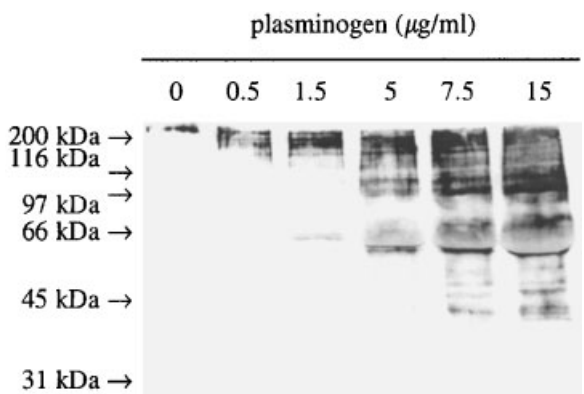

Fig. 4. Dose-dependent proteolysis of fibronectin. VSMCs were incubated for 48 h in serum-free DMEM with plasminogen (0–15 μg/ml), as indicated. Conditioned media were then analyzed by immunoblotting.

To determine if the proMMPs produced by VSMCs are activated by plasmin, Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis was measured in the presence of batimastat. This MMP inhibitor (3 μM) did not prevent the increase of substrate hydrolysis induced by plasminogen (Fig. 6). Unlike batimastat, the phosphinic peptide inhibitor Z-FΨ(PO_2CH_2)A-R-F-OH, was more efficient at preventing the time- and dose-dependent increase in Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis (Fig. 6). Inhibition was maximal after 24 h of treatment or upon the addition of 5 μg/ml plasminogen (62 and 70% inhibition, respectively). In addition, a monoclonal antibody directed against the GBD of human Fn, Mab IST10, was used. This specific anti-GBD antibody prevented the increase in substrate hydrolysis induced by plasminogen to the same extent as Z-FΨ(PO_2CH_2)A-R-F-OH (Fig. 7). No inhibition was observed when a non-specific antibody was used as a control (Fig. 7). These results show that plasmin activates a proteinase that displays similar biochemical properties to Fn-proteinase.

Fn-Proteinase Activation by Plasmin in VSMCs Conditioned Media

To determine whether plasmin activates the phosphinic peptide-sensitive Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolyzing activity in solution, media conditioned by VSMCs were treated with increasing amounts of plasmin and Fn-proteinase activity was determined. VSMCs conditioned media hydrolyzed the fluorogenic substrate after plasmin treatment (Fig. 8). This hydrolysis occurred in a dose-dependent manner, reaching a plateau with 1 μg plasmin. The activation of this enzyme requires plasmin enzymatic activity as the serine protease inhibitor, pefabloc®, prevented this hydrolysis (Fig. 8A). The hydrolysis induced by plasmin was strongly inhibited by the phosphinic peptide inhibitor (83% inhibition) (Fig. 8B). These results show that plasmin-mediated Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolyzing activity, likely to be Fn-proteinase, can occur in solution.

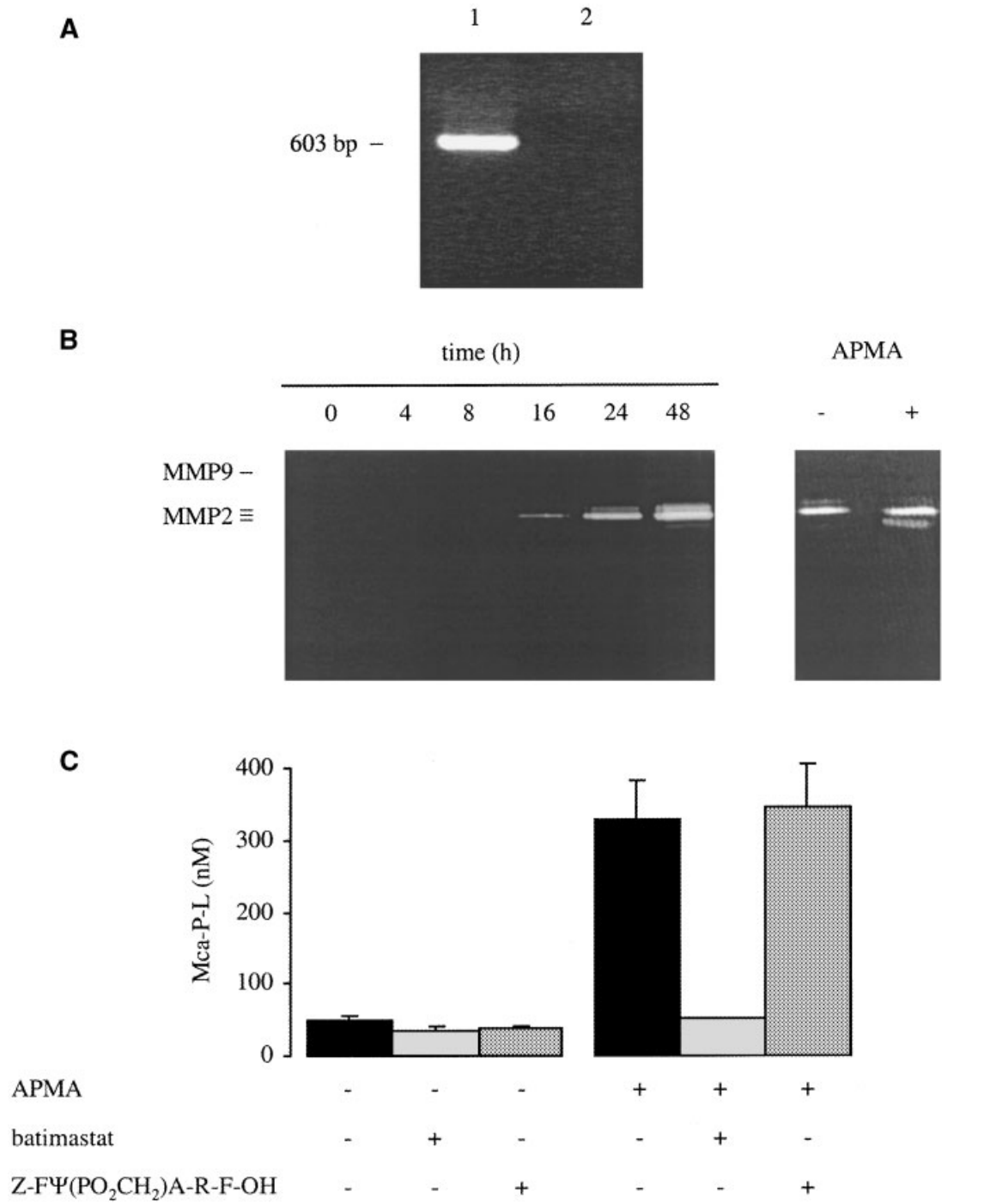

Fig. 5. VSMCs constitutively secrete proMMP-2. **A**: cDNAs obtained from VSMCs were used as templates for PCR with specific primers for rat MMP-2 (**lane 1**) and MMP-9 (**lane 2**). **B**: VSMCs were incubated in serum-free DMEM. At the indicated times, conditioned media were collected and analyzed for the presence of MMP-2 and MMP-9 (5 µl of 10-fold concentrated media) by gelatin zymography. Conditioned media of VSMCs incubated in serum-free DMEM for 48 h were incubated in the presence of APMA (1 mM) and analyzed by gelatin zymography. **C**: Conditioned media of VSMCs incubated in serum-free DMEM for 48 h were analyzed for MMP activity by measuring Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis in the absence or in the presence of batimastat (3 µM) or the phosphinic peptide inhibitor Z-FΨ(PO_2CH_2)A-R-F-OH (100 µM). The data presented are the mean ± SEM for six experiments.

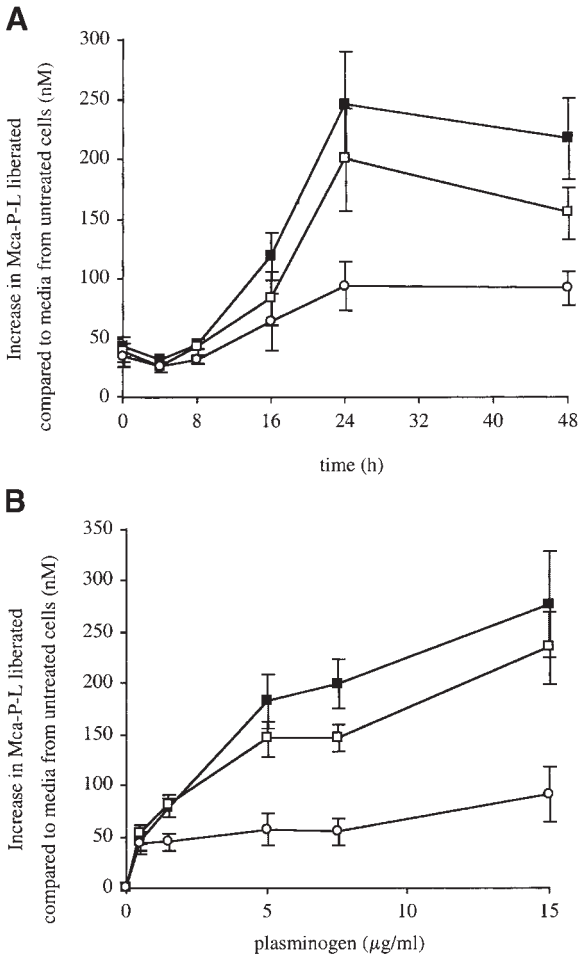

Fig. 6. Time- and dose-dependent activation of proteinase by VSMC-activated plasmin. **A**: Time-course. VSMCs were incubated in serum-free DMEM with plasminogen (7.5 μg/ml) for the indicated times. **B**: Dose-response. VSMCs were incubated for 48 h in serum-free DMEM with plasminogen (0–15 μg/ml), as indicated. At the end of experiments, conditioned media were collected and Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis was measured in the absence (■) or in the presence of batimastat (3 μM) (□) or the phosphinic peptide inhibitor Z-FΨ(PO_2CH_2)A-R-F-OH (100 μM) (O). The data are expressed as the increase in substrate hydrolysis compared to in unstimulated cells. The data presented are the mean ± SEM for three experiments performed in duplicate. Values for the hydrolysis in the unstimulated cells were: (A) 43.2 ± 1.2, 44.5 ± 1.7, 43.1 ± 1.9, 79.1 ± 12.5, 87.7 ± 2.4, and 117.0 ± 8.7 nM of Mca-P-L liberated after 0, 4, 8, 16, 24, and 48 h, respectively and (B) 136.7 ± 5.6, 112.5 ± 5.8, and 174.6 ± 6.7 nM of Mca-P-L were liberated in the control, batimastat-treated sample (3 μM) and Z-FΨ(PO_2CH_2)A-R-F-OH-treated sample (100 μM), respectively.

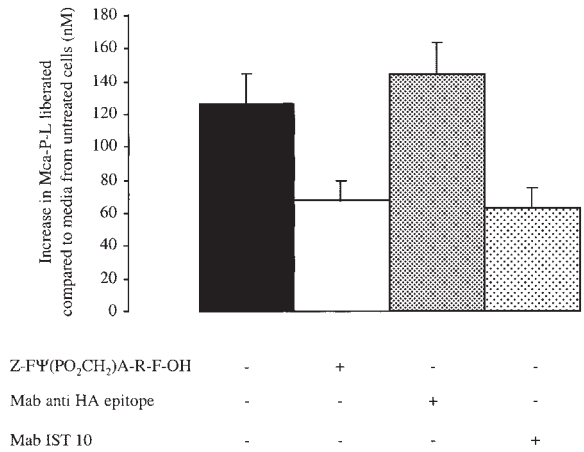

Fig. 7. Inhibition of Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis induced by VSMC-activated plasmin by a monoclonal antibody directed against the gelatin-binding domain of fibronectin. VSMCs were incubated in serum-free DMEM in the presence or absence of plasminogen (7.5 μg/ml). After 48 h, conditioned media were collected and Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis was measured with or without Z-FΨ(PO_2CH_2)A-R-F-OH (100 μM), Mab IST 10, a monoclonal antibody directed against the GBD of Fn, (5 μg) and a monoclonal antibody directed against HA epitope (5 μg). The data are expressed as the increase in substrate hydrolysis compared to in unstimulated cells. The data are presented as the mean ± SEM for five experiments. Values for the hydrolysis in the unstimulated cells were: 64.5 ± 4.8, 60.0 ± 5.2, 95.2 ± 3.6, and 54.3 ± 1.9 nM of Mca-P-L liberated for control, Z-FΨ(PO_2CH_2)A-R-F-OH, Mab anti HA epitope and Mab IST 10, respectively.

Batimastat (3 μM) inhibited 29% of the hydrolysis increase, suggesting that MMPs are weakly activated by plasmin (Fig. 8B). We then investigated the activation of proMMP-2 by increasing amounts of plasmin in vitro. Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis did not increase after treatment of proMMP-2 with plasmin. These results indicate that plasmin does not activate proMMP-2 in solution (data not shown). Thus, different MMPs may be activated by plasmin in conditioned media from quiescent VSMCs.

The plasmin-mediated activation of Fn-proteinase may be a directly mediated by plasmin or may require a preliminary MMP activation. To address this question, VSMC-conditioned media were treated with APMA, to activate MMPs, and Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis was measured. The high level of hydrolysis induced by APMA was totally inhibited by batimastat, whereas no inhibition was observed with Z-FΨ(PO_2CH_2)A-R-F-OH (Fig. 5C). This result suggests that VSMC-synthesized MMPs are not able to activate Fn-proteinase (Fig. 5C). In another set of experiments, Fn was incubated with plasmin, and the hydrolysis of Mca-P-L-G-L-Dpa-A-R-NH_2 was measured. Treatment of Fn with plasmin increased substrate hydrolysis from 53.6 ± 5.8 to 231.0 ± 14.6 nM of Mca-P-L

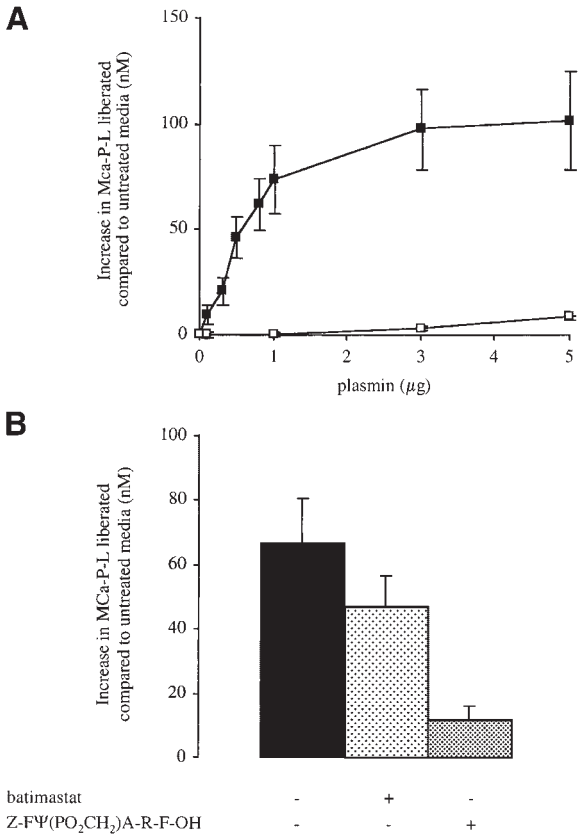

Fig. 8. Treatment of VSMC-conditioned media with plasmin activates Fn-proteinase. **A**: Dose-dependent increase in Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis induced by plasmin. Conditioned media from VSMCs cultured in serum-free DMEM for 48 h were incubated with increasing amounts of plasmin (0–5 mg) in absence (■) or in presence of pefabloc® (1 mM) (□), a general serine protease inhibitor. Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis was then determined. The data are expressed as the increase in substrate hydrolysis compared to in untreated media (120.9 ± 25.1 nM of Mca-P-L liberated) and are presented as the mean ± SEM for six experiments. **B**: Conditioned media of VSMCs cultured in serum-free DMEM for 48 h were incubated in the presence or absence of plasmin (1 μg) with or without batimastat (3 μM) and Z-FΨ(PO_2CH_2)A-R-F-OH (100 μM) and assayed for Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis. The data are expressed as the increase in substrate hydrolysis compared to in untreated media (89.8 ± 12.5, 74.6 ± 11.9, and 78.5 ± 11.2 nM of Mca-P-L liberated for control, batimastat and Z-FΨ(PO_2CH_2)A-R-F-OH, respectively). The data are presented as the mean ± SEM for seven experiments.

liberated (n = 6) (Fig. 9). The hydrolysis of Mca-P-L-G-L-Dpa-A-R-NH_2 induced by plasmin increased both in a time- and dose-dependent manner (results not shown). Substrate hydrolysis was totally inhibited by the phosphinic peptide inhibitor (96% inhibition), but not by batimastat (6% inhibition) (Fig. 9). These results show that plasmin can directly activate Fn-proteinase through Fn limited proteolysis.

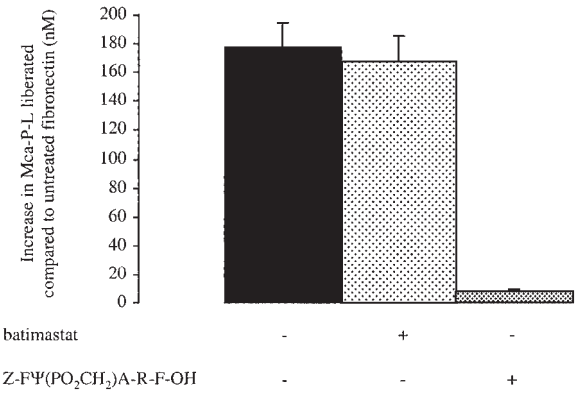

Fig. 9. In vitro processing of fibronectin by plasmin activates Fn-proteinase. Bovine Fn (10 μg) was incubated in the presence or absence of plasmin (6 μg) with or without batimastat (3 μM) and Z-FΨ(PO_2CH_2)A-R-F-OH (100 μM) and assayed for Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis. The data are expressed as the increase in substrate hydrolysis compared to untreated Fn (177.4 ± 17.9, 166.7 ± 19.1 and 7.4 ± 2.3 nM of Mca-P-L liberated for control, batimastat and Z-FΨ(PO_2CH_2)A-R-F-OH, respectively). The data presented are the mean ± SEM for six experiments.

Taken together, these results show that VSMCs convert exogenous plasminogen into plasmin. Plasmin then induces the proteolysis of ECM components and activates a proteinase that is similar to Fn-proteinase.

DISCUSSION

The plasminogen/plasmin system plays an important role in vascular remodeling [Carmeliet and Collen, 1998]. Components of this system are expressed by VSMCs and upregulated after injury [Clowes et al., 1990; Reidy et al., 1996], suggesting that VSMCs control plasmin activation in their pericellular environment. Nevertheless, the VSMC-mediated conversion of plasminogen to plasmin has never been reported. Our results show that primary cultured VSMCs efficiently convert exogenous plasminogen into plasmin in a time- and dose-dependent manner. Plasmin activity was detected when the plasminogen concentrations were lower than physiological levels. Plasmin was only generated in the presence of VSMCs, in a mechanism dependent on both membrane-bound plasminogen and PAs[2]. A similar mechanism of plasminogen activation has been described for other cell types [Hajjar et al., 1986; Silverstein et al., 1988; Meissauer et al., 1992], and this finding is consistent with the fact that VSMCs express cell surface receptors for plasminogen and PAs [Hajjar et al.,

1989; Ellis and Whawell, 1997]. Plasminogen fragments were observed in conditioned media after incubating VSMCs with plasminogen, as it is the case in several other cells lines [Falcone et al., 1994; Stathakis et al., 1997; Kassam et al., 2001]. These fragments included a 41 kDa fragment with caseinolytic activity and that formed a stable complex with α_2-antiplasmin. The appearance of this fragment in conditioned media was associated with plasmin activity. These results suggest that this fragment corresponds to a catalytically active plasmin fragment, which may be generated by plasmin autoproteolysis, as previously reported [Gately et al., 1997; Stathakis et al., 1997; Kassam et al., 2001].

Our results also show that VSMC-mediated plasmin generation activated a proteinase that can hydrolyze Mca-P-L-G-L-Dpa-A-R-NH_2, a MMP fluorogenic substrate. Surprisingly, the general MMP inhibitor, batimastat, only weakly inhibited this hydrolysis, suggesting that MMPs do not play a major role in this phenomenon. However, VSMCs are a source of MMPs in the vascular wall, and plasmin is involved in proMMPs activation [He et al., 1989; Carmeliet et al., 1997]. Quiescent VSMCs constitutively and predominantly synthesize MMP-2 in its inactive state [this study and Galis et al., 1994a]. Although the role of plasmin in the activation of proMMP-2 remains unclear, it appears that plasmin does not directly activate proMMP-2 [Okada et al., 1990; Baramova et al., 1997]. In good agreement with these previous studies, our results from experiments done in a cell-free system show that plasmin does not activate proMMP-2. However, plasmin may be involved in proMMP-2 activation in the presence of cells or cell-membrane preparations [Mazzieri et al., 1997] by acting in cooperation with the MT1-MMP/TIMP2 system [Baramova et al., 1997]. VSMCs, which are able to produce all of the components of this system, may express excess TIMP-2 compared to MT1-MMP, thus preventing proMMP-2 activation. Although VSMC-conditioned media mainly contained proMMP-2, the presence of MMPs other MMP-9 cannot be excluded as a weak batimastat-sensitive activity was observed. Plasminogen and plasmin stimulate the synthesis of MMPs in various cell lines [Berman, 1993; Gonzalez-Gronow et al., 2001]. In human VSMCs, plasminogen and plasmin stimulate both the synthesis and the activation of MMP-1 and MMP-3 [Lee et al., 1996]. However, with plasminogen concentrations used in our study, MMP proteins were induced but without any effect on their activation. Another hypothesis to explain the weak MMP activity observed is that active MMPs can be inhibited by TIMPs synthesized by VSMCs.

VSMCs synthesize their own ECM. We investigated the effects of VSMC-mediated plasmin activation on ECM integrity. Plasmin activation induced Fn proteolysis as shown by the appearance of Fn fragments in cell-conditioned media. Numerous studies have reported that some Fn fragments display biological activities not found in the parent protein. These Fn fragments, present in remodeling tissue [Xie et al., 1992; Schedin et al., 1996], can affect cell behavior and ECM integrity [Homandberg et al., 1992; Kapila et al., 1996; Grant et al., 1998].

Latent collagenase activity has been described in the GBD of Fn [Lambert Vidmar et al., 1991]. Boudjennah et al. subsequently showed that this corresponds to a zinc- dependent metalloproteinase that is activated after limited proteolysis of Fn and the release of GBD [Boudjennah et al., 1998]. Schnepel and Tschesche showed that *E. coli* can produce a recombinant Fn-fragment corresponding to the GBD, displaying collagenase activity [Schnepel and Tschesche, 2000]. This metalloproteinase, called Fn-proteinase, degrades denatured collagen I, laminin and collagen IV [Lambert Vidmar et al., 1991], and may be involved in cartilage matrix turnover [Chevalier et al., 1996]. Fn-proteinase, MMP-2 and MMP-9, all bind gelatin through their Fn-type II modules, display gelatinase activity and hydrolyze Mca-P-L-G-L-Dpa-A-R-NH_2 [Boudjennah et al., 1998], suggesting that they share common structural and functional properties. However, Fn-proteinase exhibits a specific inhibition pattern. The phosphinic peptide inhibitor directed against the EC 3.4.24.15 thimet oligopeptidase, Z-FΨ(PO_2CH_2)A-R-F-OH, considerably inhibits its activity, whereas MMP inhibitors are weakly active [Boudjennah et al., 1998]. Both Fn proteolysis and the activation of a batimastat-insensitive Mca-P-L-G-L-Dpa-A-R-NH_2 hydrolysis induced by plasmin prompted us to examine whether plasmin activates an enzyme, which may correspond to Fn-proteinase. Such enzymatic activity was not detected in conditioned media of quiescent VSMCs. The increase in Mca-P-L-G-L-Dpa-A-R-NH_2

hydrolysis induced by plasmin was strongly inhibited by the phosphinic peptide and also by a monoclonal antibody directed against the GBD of human Fn. Furthermore, phosphinic peptide-sensitive activity was observed after in vitro processing of bovine Fn by plasmin. The finding that the plasmin-mediated Fn-proteinase generation is associated with Fn proteolysis is consistent with the previously described mechanism of Fn-proteinase activation [Lambert Vidmar et al., 1991; Boudjennah et al., 1998]. In conditioned media of quiescent VSMCs, Fn was not degraded and no Fn-proteinase activity was detected, whereas plasmin induced both Fn proteolysis and Fn-proteinase activation. The generation of Fn-proteinase was prevented by inhibiting plasmin with pefabloc® or α_2-antiplasmin. These results strongly suggest that Fn-proteinase is activated through the direct proteolysis of Fn by plasmin.

This sudy is the first to describe this proteinase activation cascade involving plasminogen and Fn. Through ECM breakdown and Fn proteolysis, plasmin activates Fn-proteinase, a cryptic zinc-dependent metalloproteinase located in the GBD of Fn. The biological functions of Fn-proteinase and its proteinase cascade remain to be determined. In vitro, the Fn-proteinase degrades some ECM components [Lambert Vidmar et al., 1991] and thus may participate in the breakdown of the ECM network during vascular remodeling after injury. In addition, Fn-proteinase may regulate the activity of growth factors and proteases, as is the case for other matrix proteases. Together, these putative activities of Fn-proteinase may account for the previously reported effects of GBD on cell proliferation and migration [Schor et al., 1996; Grant et al., 1998]. Further experiments are necessary to investigate the effect of Fn-proteinase on cell behavior and cell environment.

In summary, after injury, VSMCs may regulate their pericellular environment by converting exogenous plasminogen into plasmin. Plasmin, in turn, induces the proteolysis of ECM components and activates proteinase. The localization and functions of Fn in the ECM and the identified substrates of Fn-proteinase [Lambert Vidmar et al., 1991] suggest that Fn-proteinase plays a role in vascular remodeling. Fn-proteinase may act in concert with other matrix proteinases in vascular remodeling after injury.

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