Divergent regulation by growth factors and cytokines of 95 kDa and 72 kDa gelatinases and tissue inhibitors of metalloproteinases-1, -2 and -3 in rabbit aortic smooth muscle cells

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The migration and proliferation of vascular smooth muscle cells (SMCs) during neointima formation in atherosclerosis and angioplasty restenosis is mediated by certain growth factors and cytokines, one action of which may be to promote basement-membrane degradation. To test this hypothesis further, the effects of such growth factors and cytokines on the synthesis of two basement-membrane-degrading metalloproteinases, namely the 72 kDa gelatinase (MMP-2, gelatinase A) and the 95 kDa gelatinase (MMP-9, gelatinase B) and three tissue inhibitors of metalloproteinases (TIMPs) was studied in primary cultured rabbit aortic SMCs. Expression of the 95 kDa gelatinase was increased by phorbol myristate acetate, foetal calf serum, thrombin and interleukin-1α (IL-1α); platelet-derived growth factor (PDGF) BB alone had no effect but acted synergistically with IL-1α. A selective protein kinase C inhibitor, Ro 31-8220, abolished induction of the 95 kDa gelatinase. In contrast, none of the agents tested modulated the synthesis of the 72 kDa gelatinase. We conclude that maximal up-regulation of 95 kDa gelatinase expression requires the concerted action of growth factors and inflammatory cytokines mediated, in part, by a protein kinase C-dependent pathway. TIMP-1 and TIMP-2 were highly expressed, and their synthesis was not affected by growth factors or cytokines. Expression of TIMP-3 mRNAs was, however, increased by PDGF and transforming growth factor β, especially in combination. Divergent regulation of gelatinase and TIMP expression implies that either net synthesis or net degradation of basement membrane can be mediated by appropriate combinations of growth factors and cytokines.

INTRODUCTION

Migration of smooth muscle cells (SMCs) from the tunica media to the intima, and their subsequent proliferation, occur in response to vessel injury during atherosclerosis, angioplasty restenosis and in vein grafts and leads to formation of a neointima. Several growth factors and cytokines released by circulatory cells and cells of the vessel wall may act as SMCs mitogens and chemoattractants to modulate this response [1]. In addition to their direct effects on growth signal transduction pathways, there is accumulating evidence that remodelling of the surrounding extracellular matrix (ECM) in response to these growth factors is necessary to permit proliferation and movement of SMCs. The SMCs in the normal vasculature are surrounded by a continuous basement membrane and are further embedded within a complex matrix of interstitial connective tissue [2]. Components of the basement membrane, in particular heparan sulphate proteoglycans, laminin and type IV collagen, are capable of maintaining SMCs in a quiescent and differentiated state with a low replication rate [3]. Disruption of the basement membrane is probably necessary therefore to release SMCs from natural inhibitory constraints.

The matrix-degrading metalloproteinases (MMPs) are thought to be primarily responsible for the turnover of matrix components at physiological pH. The MMP family includes the collagenases, stromelysins and gelatinases, which can interact synergistically to degrade all the ECM components. The gelatinase subgroup, which comprises a 72 kDa gelatinase A (MMP-2) and a 95 kDa gelatinase B (MMP-9), efficiently degrade type IV collagen [4], the major stuctural component of basement membranes [5]. Hence gelatinases, in particular, are implicated in removing the first ECM barrier to migration and to proliferation of SMCs. In direct support for this hypothesis, we have detected gelatinase (but not stromelysin or collagenase) activity in rabbit aortic SMCs and shown that migration and proliferation of SMCs from cultured explants is inhibited by specific, synthetic inhibitors of MMPs [6]. The effect of MMP inhibitors on migration of SMCs has recently been confirmed in the rat carotid injury model *in vivo* [7].

Release of gelatinases and other MMPs has also been demonstrated in bovine, rat, pig, baboon and human SMCs *in vitro* [8–11]. Furthermore, activities of collagenase, stromelysin and the 95 kDa gelatinase are increased in models of atherosclerosis and angioplasty restenosis [7,12–14]. The factors responsible for the up-regulation of MMPs in SMCs have not been fully defined, however. From previous work, secretion of the 95 kDa but not the 72 kDa gelatinase is induced by interleukin-1α (IL-1α), tumour necrosis factor α (TNFα) and phorbol esters, in a variety of cell types [15], including human saphenous vein SMCs [16]. However, the effects of growth factors, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and thrombin, thought to be important in neointima formation, have not been reported previously. We have therefore studied the expression of the 95 kDa and 72 kDa

Abbreviations used: SMCs, smooth muscle cells; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; ECM, extracellular matrix; PKC, protein kinase C; AP-1, activator protein-1; NF-κB, nuclear factor κB; PMA, phorbol 12-myristate 13-acetate; FCS, foetal calf serum; IL-1α, interleukin-1α; PDGF, platelet-derived growth factor; TGFβ, transforming growth factor β; bFGF, basic fibroblast growth factor; TNFα, tumour necrosis factor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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gelatinases at both the protein and mRNA levels in response to these growth factors alone and in combination with the cytokines, IL-1α, TNFα and transforming growth factor β (TGFβ).

Endogenous tissue inhibitors of MMPs (TIMPs) bind and inactivate the secreted MMPs. Differential regulation of MMP and TIMP production is therefore presumed necessary to allow net matrix degradation [17]. We have therefore studied also the regulation by growth factors and cytokines of the three TIMPs that have been isolated and cloned, TIMP-1 [18], TIMP-2 [19] and TIMP-3 [20].

MATERIALS AND METHODS

Materials

Human HT-1080 fibrosarcoma cells were obtained from the European Collection of Cell and Animal Cultures (Salisbury, Wilts., U.K.). Phorbol 12-myristate 13-acetate (PMA) and thrombin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Human recombinant PDGF BB and TGFβ were purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). bFGF was purchased from Promega (Southampton, U.K.). Human recombinant IL-1-α and TNFα were generous gifts from Hoffman-La Roche (Basle, Switzerland), and the protein kinase C (PKC) inhibitor, Ro 31-8220 (3-{1-[3-(amidinothio)propyl]-3-indolyl}-4-(1-methyl-3-indolyl)-1*H*-pyrrole-2,5-dione methylsulphonate), was a generous gift from Roche Products (Welwyn Garden City, Herts., U.K.). Purified human TIMPs-1, -2 and -3 were generously provided by Dr. G. Murphy (Strangeways Research Laboratories, Cambridge, U.K.). Plasmids containing cDNAs for human 72 kDa gelatinase and TIMP-2 were obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.). Plasmids containing cDNAs for human 95 kDa gelatinase and for TIMP-3 were generous gifts from Professor K. Tryggvason (Oulu, Finland) and Dr. Carlos López-Otín (Universidad de Oviedo, Spain) respectively. The PCR (see below) was used directly to generate partial cDNAs for human TIMP-1 (from HT-1080 cDNA) and rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (from rat liver cDNA, a gift from H. Glazebrook, Department of Surgery, University of Leicester, U.K.). The sources of the other agents are indicated below in the text.

Cell culture

Medial explants were prepared from the thoracic aortas of healthy New Zealand White rabbits (approximate age 9 weeks) as previously described [21]. Briefly, explants were maintained in complete medium composed of Dulbecco's modified Eagle's medium containing penicillin–streptomycin (100 units/ml and 100 μg/ml respectively), 1 mM sodium pyruvate, 8 mM L-glutamine and 15 % (v/v) foetal calf serum (FCS; Advanced Protein Products, Brierley Hill, U.K.). After 10–14 days, SMCs were subcultured by trypsin/EDTA treatment and plated at a density of 5×10^4 cells/ml into 24-well culture plates for zymography or 75 cm^2 flasks for RNA studies and used between passages 1–3. For all experiments, subconfluent cells were rendered quiescent by washing twice in PBS followed by incubation in serum-free Dulbecco's modified Eagle's medium supplemented with 0.25 % (v/v) lactalbumin hydrolysate (Gibco BRL, Paisley, Scotland) for 3 days. Cultures were then exposed to fresh serum-free medium containing the appropriate concentration of the agent under investigation. After times from 4 to 24 h, conditioned media were removed and in some cases the cells were then extracted with perchloric acid and the ATP and DNA concentrations in the extracts were measured as previously described [21]. The DNA concentration did not vary significantly 24 h after any treatment (results not shown) and hence no correction was made for cell number when comparing conditioned media (see below). ECMs were prepared from 75 cm^2 flasks of SMCs cultures. Firstly, the supernatant was discarded and the cells washed twice in PBS. Cells were then lysed with 0.5 % Triton X-100 in phosphate buffer (pH 7.4) for 15 min, followed by a 10 min incubation in 25 mM NH_4OH to remove the nuclei and cytoskeleton. Cells were washed twice in sterile water, and the matrix was extracted with 1 ml of 0.1 % SDS dissolved in 10 mM Tris/HCl, pH 7.6.

HT1080 cells were cultured in Eagle's modified essential medium/10 % (v/v) FCS and used as a positive control for gelatinase and TIMP expression.

Zymography

Gelatinase activity was detected in conditioned media as previously described [6]. Briefly, 10 μl aliquots of conditioned media were mixed with an equal volume of non-reducing Laemmli sample buffer [22] and electrophoresed at 4 °C in SDS/7.5 % polyacrylamide gels containing 2 mg/ml gelatin derived from calf skin collagen (type III; Sigma). After electrophoresis, the gels were cleared of SDS by incubating for 1 h with two changes of 2.5 % (v/v) Triton X-100. Gels were then incubated overnight in substrate buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 10 mM $CaCl_2$ and 0.05 % Brij 35) at 37 °C. The gels were then stained with 0.1 % Coomassie Brilliant Blue, and gelatinolytic activity was revealed as clear bands against a blue-stained background. Gelatinolytic bands were size calibrated with a high-molecular-mass standard mixture of proteins (Sigma).

Reversed zymography was used to detect TIMP-1 and TIMP-2 activities in conditioned media and TIMP-3 activities in ECM extracts as described by Ward et al. [23]. Briefly, samples were concentrated using Centricon-10 concentrators (Amicon, Stonehouse, Gloucs., U.K.). The protein concentration in ECM extracts was determined with bicinchoninic acid using a kit (BCA-1; Sigma), according to the manufacturer's instructions. Samples were then applied to SDS/11 % polacrylamide gels containing 1.5 mg/ml gelatin. The standard zymographic method was modified after the removal of SDS from the gels by incubating the gels for 2 h at 37 °C in conditioned medium from PMA-activated rabbit skin fibroblasts, which provided a source of activated metalloproteinases. The gels were then incubated and stained in the same way as standard zymograms. Protection of the gelatin in the gels by the presence of TIMPs led to the production of relatively dark bands against a lighter background. Purified human TIMPs were used as standards. Reversed zymograms were subjected to densitometric analysis using a Bio-Rad (Hemel Hempstead, Herts., U.K.) GS690 imaging densitometer and associated software. Bands were identified manually, and the absorbance was integrated over the area of each band and was corrected for the background colour measured over a neighbouring region of the same gel. Paired comparisons of different treatments were made from lanes on the same gels using ANOVA for multiple comparisons and the Student's *t* test for pairs.

Preparation of RNA probes for hybridization

PCR was used to generate templates for transcription *in vitro* of antisense RNA. Primers were designed based on the published sequences within regions showing least similarity with other related genes. These were for the 72 kDa gelatinase [24] sense (5′-ACATTGACCTTGGCACC-3′) and antisense (5′-GGCATC-TGCGATGA-3′); for the 95 kDa gelatinase [4] sense (5′-

TGGGAACCAGCTGTAT-3′) and antisense (5′-ACTGCAAAGCAGGAC-3′); for TIMP-1 [18] sense (5′-ACCCACAGACGGCCTTCTGCAATTC-3′) and antisense (5′-GGCTATCTGGGACCGCAGGGACTGC-3′); for TIMP-2 [19] sense (5′-TGCAATGCAGATGTAGTGATCAGGG-3′) and antisense (5′-TGCTTATGGGTCCTCGATGTCGAGA-3′); for TIMP-3 [20] sense (5′-CTACACCATCAAGCAGATGAAGATG-3′) and antisense (5′-TGTGGCATTGATGATGCTTTTATCC-3′); and for GAPDH [25] sense (5′-ACCCCTTCATTGACCTCAACTACA-3′) and antisense (5′-ATGACCACACAGTCCATGCCATCACT-3′). These primers were used to amplify products from plasmids containing the relevant cDNAs or directly from reverse-transcribed mRNA (see above). The identity of the PCR products was established by four criteria, namely predicted size, restriction-enzyme mapping, partial dideoxysequence analysis [26] and hybridization to mRNA sequences of characteristic sizes from SMCs and HT1080 cells. In early experiments with the 72 kDa gelatinase, 92 kDa gelatinase and GAPDH, an upstream clamp (CCAC) and T7 RNA polymerase site (5′-TAATACGACTCACTATAGGGAGA-3′) were included on the 5′ end of the antisense primer, and the PCR products were then used directly as templates for transcription *in vitro* using [α-^{32}P]CTP (specific radioactivity 400 Ci/mmol; Amersham International, Amersham, U.K.) according to the manufacturer's instructions (Promega). In later experiments, for convenience, the PCR products were subcloned into the PCR II vector system (Invitrogen; Rand D Systems, Abingdon, U.K.), which contains flanking T7 and SP6 transcription sites, using the manufacturer's protocol. Following restriction-enzyme digestion, depending on orientation of the insert, with either *Spe*I or *Eco*RV restriction endonucleases (sites located at either side of the cloning site), riboprobes were generated in the presence of [α-^{32}P]CTP, using SP6 or T7 polymerase, respectively, according to the manufacturer's instructions (Promega).

Total RNA isolation and Northern blotting

Total cellular RNA was prepared from cultures as described by Chomczynski and Sacchi [27] and quantified by absorbance at 260 nm. RNA (15 μg) was fractionated on 1% agarose/2.2 M formaldehyde gels and transferred on to Hybond-N membranes (Amersham) by capillary blotting using 20 × SSC (3 M sodium chloride and 300 mM sodium citrate). The RNA was then bound by cross-linking the membrane with UV irradiation (Stratalinker; Stratagene, Cambridge, U.K.) and stained with 0.03% Methylene Blue in 0.3 M sodium acetate, pH 5.2, to assess RNA integrity and equality of loading and transfer [28].

Prehybridization of the blots was carried out at 60 °C in a buffer containing 50% (v/v) formamide, 0.25 M sodium phosphate, pH 7.2, 0.25 M NaCl, 1 mM EDTA and 7% (w/v) SDS for 15 min. Blots were subsequently hybridized in the same buffer overnight with [α-^{32}P]CTP-labelled RNA probes. Posthybridization was carried out at 65 °C using PSE buffer (0.25 M sodium phosphate, pH 7.2, 2% SDS and 1 mM EDTA) for 30–60 min followed by higher stringency washes with PES buffer (0.04 M sodium phosphate, pH 7.2, 1% SDS and 1 mM EDTA) at 65–90 °C. Blots were then exposed to Kodak XAR-5 film at 70 °C for 24–72 h. Rehybridization of membranes was carried out after stripping of residual radioactivity according to the manufacturer's instructions (Amersham).

RESULTS

Phorbol ester and serum increase expression of 95 kDa but not 72 kDa gelatinase

PMA, a direct activator of PKC and a mitogen for rabbit SMCs [29,30], increases 95 kDa gelatinase activity in many cells, including SMCs from other species [4,9]. This prompted us to investigate its effects on gelatinase expression in rabbit SMCs. Zymography revealed that PMA increased secretion of the 95 kDa but not the 72 kDa gelatinase (Figure 1, left panel). Northern-blot analysis showed that the 95 kDa and 72 kDa gelatinase probes hybridized to distinct mRNA transcripts of characteristic sizes 2.8 kb and 3.1 kb, respectively, in rabbit SMCs (Figure 1, right panel) and in HT1080 cells (results not shown), confirming their specificity. PMA increased steady-state levels of 95 kDa but not 72 kDa gelatinase mRNA (Figure 1, right panel), which corresponded to the pattern of protein secretion. The mRNA levels of the house-keeping control gene, GAPDH, remained constant (Figure 1, right panel).

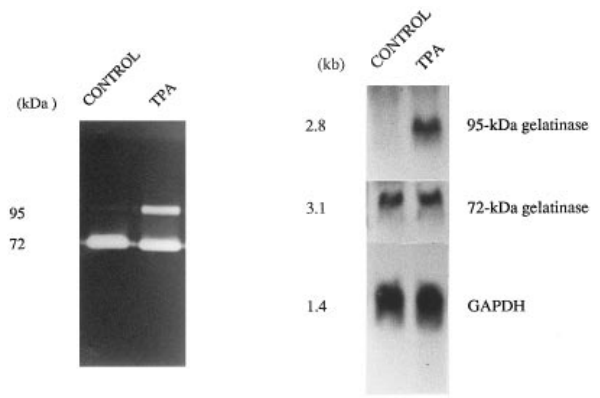

Figure 1 Effect of PMA on gelatinase production

SMCs were grown to subconfluence, transferred to serum-free medium for 72 h and then treated with 10 nM PMA (≡TPA) for 12 h. (Left panel) Conditioned media were collected and 10 μl aliquots were subjected to gelatin zymography. The relative molecular masses of the proteins are indicated in kDa. (Right panel) RNA was extracted from the cells and the resulting Northern blot was probed with riboprobes for 95 kDa gelatinase, 72 kDa gelatinase and GAPDH as a loading control. The transcript sizes of each enzyme are denoted in kb. The panels shown are representative of four experiments.

We next investigated a more physiological inducer of SMC proliferation, FCS. Northern-blot analysis revealed a transient induction of 95 kDa gelatinase mRNA by FCS, peaking within 6 h and then declining up to 24 h. No consistent variation was observed in 72 kDa gelatinase mRNA levels (Figure 2, upper panel). Owing to the interference of FCS in zymographic analysis, we were unable to investigate whether FCS affected 95 kDa gelatinase secretion under the same experimental conditions. Instead, primary SMCs were cultured for 6 days, with FCS present for the first 4 days of the culture followed by serum-free medium for 2 days. The secretion of the 95 kDa gelatinase into the conditioned medium was then compared with conditioned medium collected over the the last 2 days of cultures grown in the absence of FCS for 6 days. The results confirmed that FCS increased secretion of the 95 kDa but not the 72 kDa progelatinase (Figure 2, lower panel).

Induction of 95 kDa gelatinase expression – synergistic action of PDGF and IL-1α

Since cytokines, such as IL-1α and TNFα, and growth factors, such as PDGF and TGFβ, are present at sites of vascular injury and may promote SMC proliferation, we analysed their effects on 95 kDa and 72 kDa gelatinase expression. Zymography revealed that exposure of SMCs to either PDGF BB or IL-1α

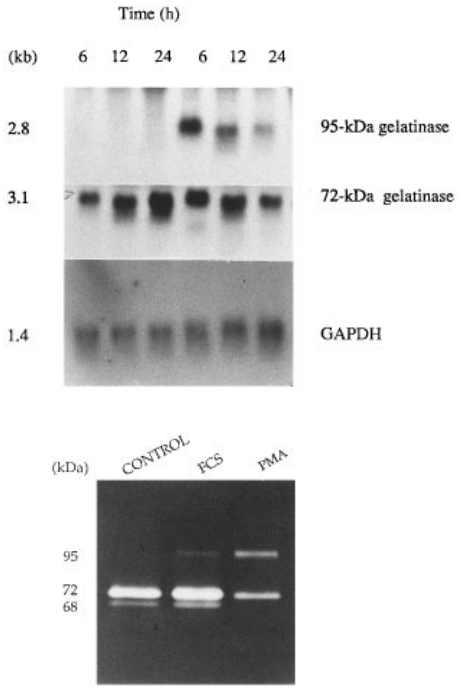

Figure 2 Effect of FCS on gelatinase production

(Upper panel) SMCs were grown to subconfluence in 75 cm^2 flasks, transferred to serum-free medium for 72 h and then treated with 10% FCS. Cells (approximately 10^6) were harvested for RNA at the times indicated and subjected to Northern-blot analysis. Lanes 1–3 show control cultures (absence of FCS); lanes 4–6 show FCS-treated cultures. The blot was probed with riboprobes for 95 kDa gelatinase, 72 kDa gelatinase and GAPDH as a loading control. (Lower panel) SMCs were cultured in 96-well plates for 6 days in the absence of serum (lane 1) or for 4 days in the presence of 15% FCS and then continued in serum-free medium for a further 2 days (lane 2). Conditioned medium was then collected and subjected to gelatin zymography. Conditioned medium from PMA-treated smooth muscle cells (lane 3) was also subjected to zymography for comparison. These results are representative of three experiments.

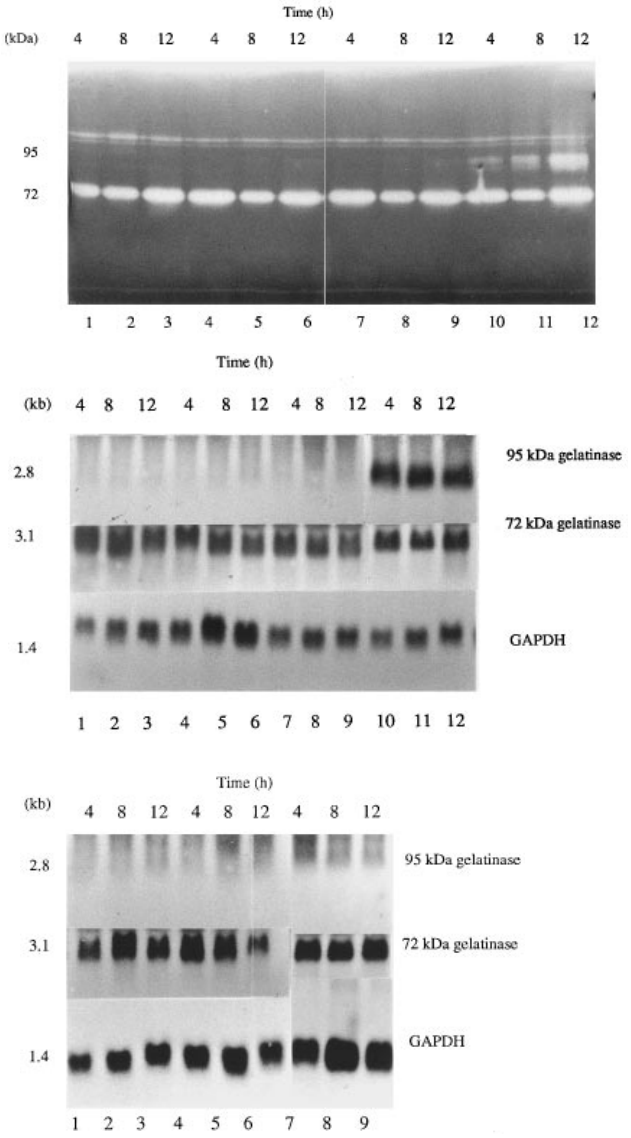

Figure 3 Effect of PDGF, TGFβ and IL-1α on gelatinase production

SMCs were grown to subconfluence in 75 cm^2 flasks, transferred to serum-free medium for 72 h and then treated with either 20 ng/ml PDGF, 10 ng/ml IL-1α, 5 ng/ml TGFβ, 10 ng/ml TNF or combinations of these agents as indicated below and for the times shown. (Upper panel) Conditioned media were collected and 10 μl aliquots were subjected to gelatin zymography. Lanes 1–3 show unstimulated cells; lanes 4–6 show cells stimulated with PDGF; lanes 7–9 show cells stimulated with IL-1α; and lanes 10–12 show cells stimulated with PDGF + IL-1α. The molecular masses of the protein bands obtained by interpolation of standards (not shown) are indicated in kDa. (Middle panel) RNA was extracted from the same cells, and the resulting Northern blots were probed with riboprobes for either 95 kDa gelatinase, 72 kDa gelatinase or GAPDH. The transcript sizes of each enzyme are denoted in kb. Lanes 1–3 show unstimulated cells; lanes 4–6 show cells stimulated with PDGF; lanes 7–9 show cells stimulated with IL-1α; and lanes 10–12 show cells stimulated with PDGF + IL-1α. (Lower panel) Similar Northern blots to the middle panel. Lanes 1–3 show untreated cells; lanes 4–6 show cells treated with TGFβ; and lanes 7–9 show cells treated with PDGF + TGFβ. The data are representative of three similar experiments.

alone for up to 12 h did not stimulate 95 kDa gelatinase secretion. However, increased 95 kDa gelatinase secretion was detected as early as 4 h after addition of a combination of PDGF and IL-1α. Secretion of 95 kDa gelatinase increased in a time-dependent manner up to 12 h (Figure 3, upper panel), whereas secretion of 72 kDa gelatinase was unaltered (Figure 3, upper panel). These findings were confirmed by Northern-blot analysis (Figure 3, middle panel), which showed that 95 kDa but not 72 kDa gelatinase mRNA levels were dramatically increased at 4 to 12 h by PDGF and IL-1α together but not by either PDGF or IL-1α alone. Incubation of rabbit aortic SMCs for longer periods (i.e. 24 h) in the presence of IL-1α alone led to modestly increased secretion of 95 kDa gelatinase (Figure 4). PDGF was ineffective on its own but greatly potentiated the effect of IL-1α.

Other growth factors for SMCs include bFGF [31], thrombin [32], and 5-hydroxytryptamine [33]. We investigated their effect on gelatinase production also. Zymography demonstrated that bFGF, like PDGF, had no effect on gelatinase secretion (Figure 5). 5-Hydroxytryptamine alone also had no effect (results not shown). By contrast, thrombin alone stimulated 95 kDa gelatinase secretion, and the effect of thrombin was slightly potentiated by bFGF (Figure 5). No changes in 72 kDa gelatinase expression were detected under the same conditions (Figure 5).

TNFα alone increases 95 kDa gelatinase secretion from human saphenous vein SMCs [16]. It was ineffective, however, even over 24 h in rabbit aortic SMCs (results not shown). TGFβ has been reported either to inhibit or to stimulate SMC proliferation and to induce 95 kDa gelatinase expression in other cell types [34,35]. In rabbit SMCs, however, zymography (results not shown) and Northern-blot analysis (Figure 3, lower panel) revealed that TGFβ alone had no effect on 95 kDa gelatinase mRNA ex-

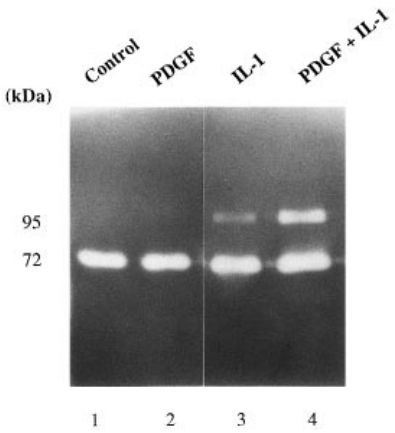

Figure 4 Effect of PDGF and IL-1α over 24 h

Zymographic analysis of 10 μl aliquots of conditioned media collected following 24 h exposure of SMCs to either PDGF, IL-1α or PDGF + IL-1α at the above stated concentrations. The results are representative of six separate experiments.

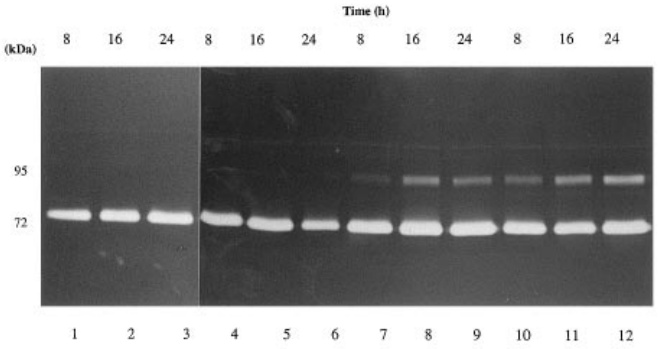

Figure 5 Effect of thrombin and bFGF on gelatinase secretion

SMCs were grown to subconfluence in 24-well dishes, transferred to serum-free medium for 72 h and then treated with 2 units/ml thrombin, 20 ng/ml bFGF or a combination of thrombin and bFGF. Conditioned medium was collected at the times indicated and analysed by gelatin zymography. Lanes 1–3 show untreated cultures; lanes 4–6 show cultures treated with bFGF alone; lanes 7–9 show cells treated with thrombin; and lanes 10–12 show cells treated with thrombin + bFGF. These results are representative of two separate experiments.

pression, consistent with the previous report in human SMCs [16]. When added simultaneously with PDGF, TGFβ weakly induced 95 kDa gelatinase mRNA expression (Figure 3, lower panel) but did not stimulate measurable protein secretion over 24 h (results not shown).

PKC activation is required for stimulation of 95 kDa gelatinase secretion by PDGF and IL-1α

A selective PKC inhibitor, Ro 31-8220, was used to investigate whether PKC activation is necessary for the induction of 95 kDa gelatinase by PDGF and IL-1α. Zymography demonstrated a dose-dependent inhibition of 95 kDa gelatinase secretion, which was complete with 3 μM Ro 31-8220 (Figure 6). A similar inhibitory potency of Ro 31-8220 has been reported against proliferation of rabbit aortic SMCs [29]. To ascertain whether the inhibitory effect of Ro 31-8220 was due to cytotoxicity, the ATP concentrations in the cell extracts were measured and found not to vary from the control (legend to Figure 6). Secretion of

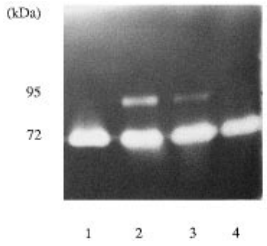

Figure 6 Effect of PKC inhibition on gelatinase secretion

SMCs cultured in serum-free medium for 72 h were pretreated for 10 min in the presence or absence of varying concentrations of the PKC inhibitor, Ro 31-8220, and then 20 ng/ml PDGF + 10 ng/ml IL-1α was added. After 24 h, conditioned media were collected and analysed by gelatin zymography. Lane 1 shows untreated cells; lane 2 shows cells treated with PDGF + IL-1α; and lanes 3 and 4 show cells treated with PDGF + IL-1α in the presence of Ro 31-8220 at 1 μM and 3 μM respectively. ATP concentrations (nmol/ml) in trichloroacetic acid extracts of cells were, for untreated cells, 0.70, and, for cells treated with PDGF + IL-1α plus 0.1% DMSO, 0.65, plus 1 μM Ro 31-8220, 0.67 or plus 3 μM Ro 31-8220, 0.69. The results are representative of three experiments.

72 kDa gelatinase was unaltered by up to 3 μM of Ro 31-8220 (Figure 6), which implies also that cell viability was unaffected.

Divergent regulation of TIMPs in rabbit SMCs

Reversed zymography demonstrated constitutive secretion of TIMP-1 and lower levels of TIMP-2 proteins. By densitometry of reverse zymograms, TIMP-1 activity was slightly increased by

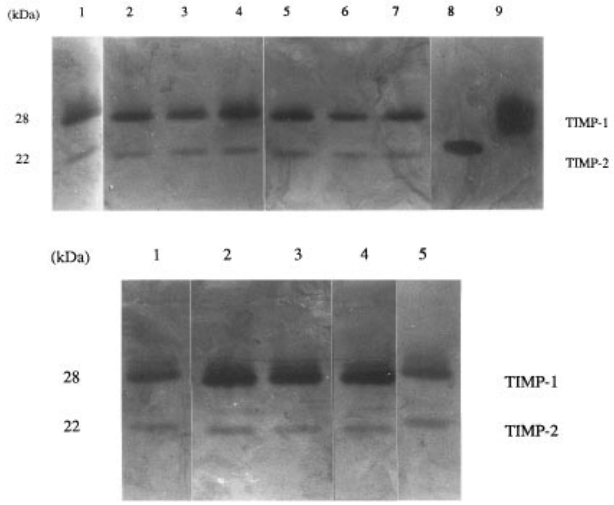

Figure 7 Effect of mediators on TIMP-1 and -2 secretion from SMCs

SMCs in serum-free medium were treated with 20 ng/ml PDGF, 10 ng/ml IL-1α, 10 ng/ml TNFα, 5 ng/ml TGFβ, 10 mM PMA or combinations of these agents as indicated below. Conditioned media were collected and concentrated 50-fold, then analysed by reversed zymography as detailed in the Materials and methods section. (Upper panel) Media obtained after 8 h of exposure to the test agents. Lane 1 shows unstimulated cells; lane 2 shows cells stimulated with PDGF + IL-1α; lane 3 shows cells stimulated with IL-1α; lane 4 shows cells stimulated with PDGF; lane 5 shows cells stimulated with PDGF + TGFβ; lane 6 shows cells stimulated with TGFβ; lane 7 shows cells stimulated with TNFα; lane 8 shows 25 ng of purified human TIMP-2; and lane 9 shows purified human TIMP-1. The relative molecular masses of the proteins are indicated in kDa. (Lower panel) Media obtained after 24 h of exposure to the test agents. Lane 1 shows unstimulated cells; lane 2 shows cells stimulated with PDGF + IL-1α; lane 3 shows cells stimulated with IL-1α; lane 4 shows cells stimulated with PDGF; and lane 5 shows cells stimulated with PMA.

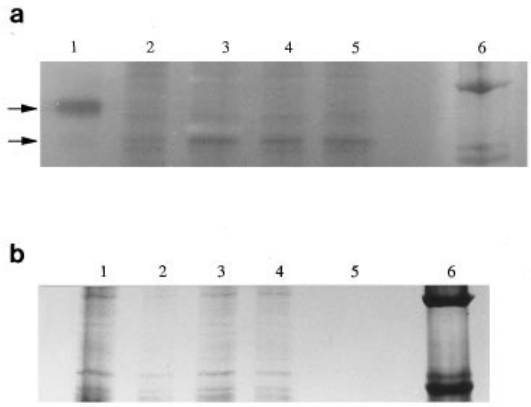

Figure 8 Effect of PDGF and TGFβ on TIMP-3 secretion from SMCs

SMCs in serum-free medium were treated with 20 ng/ml PDGF, 5 ng/ml TGFβ or combinations of these agents for 24 h as indicated below. After removing the conditioned medium, the cells were lysed with detergent and the remaining ECM was extracted and concentrated 20-fold. The protein concentration in the extracts was measured, and equal amounts of protein were then loaded on to SDS/11% polyacrylamide gels containing gelatin (**a**). The presence of TIMP-3 was detected by reversed zymography as detailed in the Materials and methods section. Lane 1 shows 75 ng of purified human TIMP-3 protein; lane 2 shows ECM from unstimulated cells; lane 3 shows ECM from cells treated with PDGF; lane 4 shows ECM from cells treated with TGFβ; lane 5 shows ECM from cells treated with PDGF + TGFβ; and lane 6 shows high-molecular-mass marker proteins. The upper and lower arrows indicate the positions of glycosylated and unglycosylated TIMP-3 activities respectively. (**b**) Equivalent amounts of ECM extract as detailed in (**a**) were analysed by electrophoresis in SDS/11% polyacrylamide gels without gelatin. Lane 1 shows ECM from unstimulated cells; lane 2 shows ECM from cells treated with PDGF; lane 3 shows ECM from cells treated with TGFβ; lane 4 shows ECM from cells treated with PDGF + TGFβ; lane 5 shows purified human TIMP-3 protein; and lane 6 shows high-molecular-mass marker proteins.

PDGF ($59 \pm 25\%$, $n = 5$, $P < 0.02$) and similar, although non-significant, increases were observed for IL-1α ($40 \pm 23\%$) and PDGF + IL-1α ($60 \pm 29\%$). TIMP-1 activity was not affected by the presence of PMA, TNFα, TGFβ, TNFα + IL-1α or TGFβ + PDGF (Figure 7). None of the agents listed above affected TIMP-2 activities (Figure 7).

Since TIMP-3 is known to be sequestered in the ECM [36], reversed zymograms of conditioned medium from SMCs (Figure 7) did not show any TIMP-3 protein. TIMP-3 protein was detected, however, in extracts of ECM from SMCs (Figure 8a). The running position of rabbit TIMP-3 was identical to the minor unglycosylated form of purified recombinant human TIMP-3, implying that the TIMP-3 recovered from the ECM of rabbit SMCs is largely unglycosylated. A constitutive protein band was seen by standard SDS/PAGE at a slightly lower molecular mass than TIMP-3 (Figure 8b), and this was visible running just ahead of the TIMP-3 bands in the reverse zymogram (Figure 8a). There were no protein bands corresponding to TIMP-3, however, in standard SDS/polyacrylamide gels, owing to its presence at levels below the detection limit for Coomassie Brilliant Blue staining. This was confirmed by the absence of a band for the TIMP standard in SDS/PAGE (Figure 8b, lane 5). These experiments confirm that the bands corresponding to TIMP-3 in Figure 8(a) arise from its ability to inhibit gelatinase. The levels of TIMP-3 measured by reverse zymography were consistently increased by PDGF and TGFβ on their own (Figure 8a). The combination of PDGF and TGFβ was no more effective than PDGF alone (Figure 8a).

Northern-blot analysis demonstrated that the TIMP-1 riboprobe hybridized very strongly to the expected 0.9 kb mRNA transcript in untreated SMCs cultures (Figure 9, left panel). Expression of TIMP-1 mRNA was not altered in the presence of growth factors added either alone or in combination (Figure 9). The TIMP-2 probe hybridized to two recognized mRNA species of 3.5 and 1.0 kb with equal intensities, and expression was again not significantly altered by the presence of growth factors or cytokines (Figure 9). In contrast to both TIMPs -1 and -2, TIMP-3 exhibited very low mRNA expression in untreated cultures. However, the probe hybridized to three previously described transcripts of 5, 2.8 and 1.1 kb in response to TGFβ or PDGF, with levels of each transcript increasing up to 12 h (Figure 9, left panel) and further increasing to 24 h (results not shown). The induction of TIMP-3 mRNA was augmented when PDGF and TGFβ were added simultaneously (Figure 9, right panel). IL-1α and TNFα alone had no effect on TIMP-3 mRNA levels but weakly enhanced expression when added together (Figure 9, right panel). The 5.0 kb band was the predominant TIMP-3 transcript under all the conditions tested. These results demonstrated a divergent pattern of regulation for TIMP-3 compared with TIMPs-1 and -2. They also showed divergent regulation of all the TIMPs compared with the 95 kDa gelatinase.

DISCUSSION

Induction of 95 kDa gelatinase by growth factors and cytokines

Increased expression of 95 kDa gelatinase occurs in human atherosclerotic plaques compared with normal arteries [13], in the experimental lesions of cholesterol-fed rabbits [37] and in carotid arterial tissues of rats and pigs subjected to angioplasty [7,14,38]. The factors responsible for upregulating 95 kDa gelatinase activity in SMCs are not known, however. Previous studies have focussed on the role of inflammatory cytokines [16], but the action of growth factors and other cytokines, such as TGFβ, known to be present locally in atherosclerotic and restenotic lesions has not been previously studied systematically. We showed that prolonged exposure to IL-1α up-regulates 95 kDa gelatinase secretion, consistent with previous work on human venous SMCs [16]. By contrast, growth factors for rabbit SMCs, including PDGF, bFGF and 5-hydroxytryptamine, were not similarly effective on their own. Most interestingly, however, combinations of PDGF and IL-1α caused a synergistic up-regulation of 95 kDa gelatinase mRNA and protein expression. This original finding is likely to be of pathophysiological significance. Both PDGF and IL-1α are produced by SMCs [39,40], as well as other cell types present in atherosclerotic lesions, including endothelial cells and macrophages [41,42]. There is also direct evidence that PDGF and IL-1α are expressed and secreted locally after balloon injury and in atherosclerotic plaques [43–46]. Furthermore, PDGF and IL-1α have been shown to act in synergy to increase SMC proliferation *in vitro* [47], even though IL-1α may elicit its effects, in part, by inducing PDGF expression [48]. Our results imply that up-regulation of 95 kDa gelatinase may be a factor contributing to this synergistic stimulation of SMC proliferation.

The other agent that we found capable of inducing 95 kDa gelatinase on its own was thrombin. Thrombin is mitogenic for SMCs on its own [32], and administration of thrombin inhibitors and antagonists has been found to reduce intimal thickening after angioplasty in hypercholesterolaemic rabbits [49,50] and in a baboon arterial injury model [51]. This study implies that induction of 95 kDa gelatinase secretion by thrombin may contribute to its mitogenic potential. Furthermore, thrombin present in atherosclerotic vessels and after angioplasty may be one factor that leads to pathophysiological up-regulation of 95 kDa gelatinase secretion.

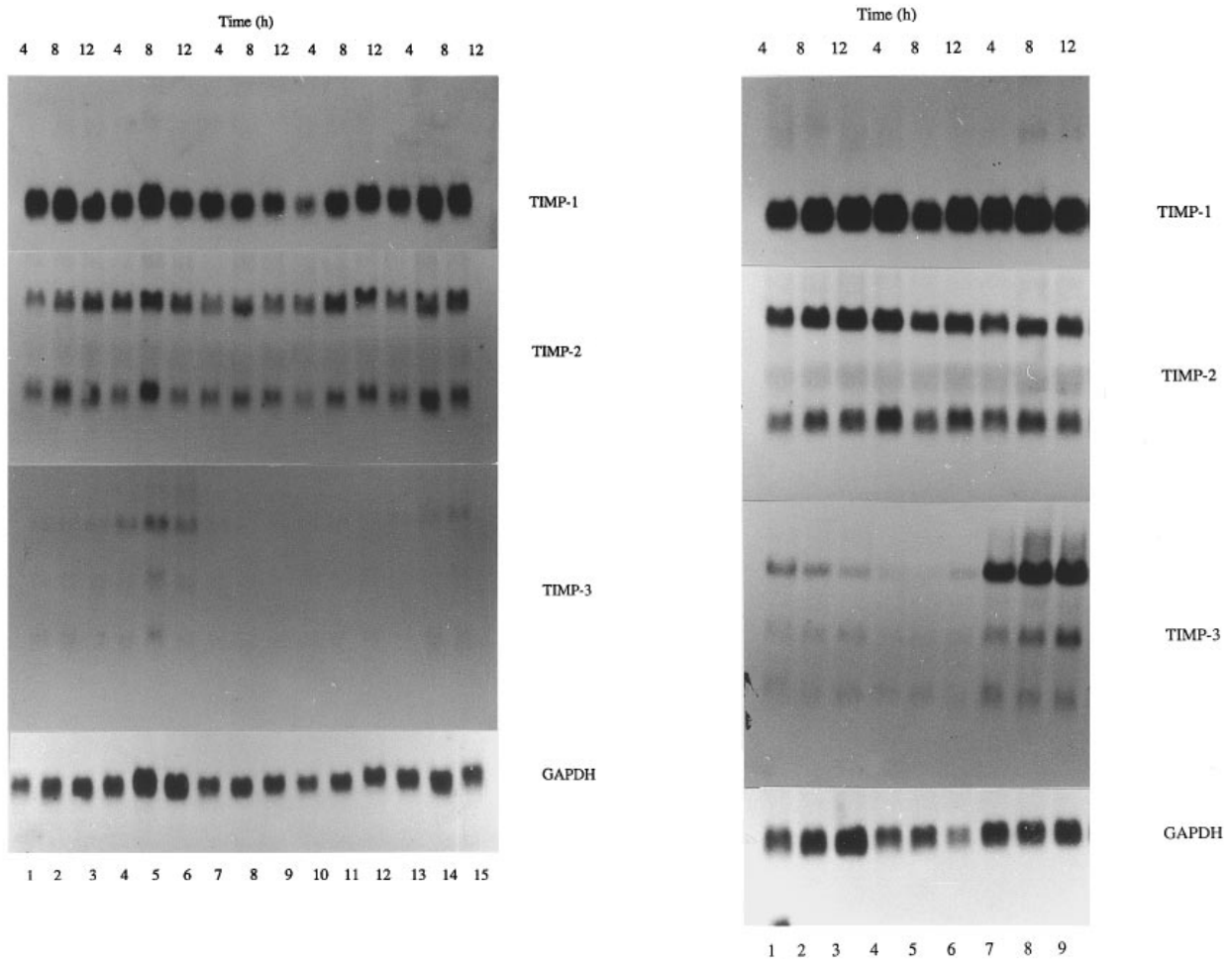

Figure 9 Effect of mediators on TIMP mRNA expression

RNA was extracted from the cells at the times indicated and Northern-blot analysis performed. Blots were hybridized with riboprobes for TIMP-1, TIMP-2, TIMP-3 or GAPDH. (Left panel) Lanes 1–3 show unstimulated cells; lanes 4–6 show cells treated with PDGF; lanes 7–9 show cells treated with IL-1α; lanes 10–12 show cells treated with TNFα; and lanes 13–15 show cells treated with TGFβ. (Right panel) Lanes 1–3 show cells treated with PDGF; lanes 4–6 show cells treated with PDGF + IL-1α; lanes 7–9 show cells treated with TNFα + IL-1α; and lanes 10–12 show cells treated with PDGF + TGFβ.

Mechanisms responsible for 95 kDa gelatinase induction

Synergistic activation of 95 kDa gelatinase expression by IL-1α and PDGF could be explained by concerted activation of more than one transduction pathway. There are binding sites for both activator protein-1 (AP-1) and nuclear factor κB (NF-κB) transcription factors in the human and murine 95 kDa gelatinase gene promoter regions [52,53], and recent studies have confirmed their presence in the conserved regions of the rabbit gene promotor [54]. Mutation analysis of the human promotor shows that both sites are essential for full induction of the enzyme. Moreover, induction of the 95 kDa gelatinase gene in fibroblast cell lines by PMA and TNFα is accompanied by the nuclear binding of both transcription factors to their cognate enhancer elements [52]. We confirmed that phorbol ester, a direct and prolonged activator of PKC, induces 95 kDa gelatinase expression in rabbit SMCs. Previous reports have demonstrated that non-specific PKC inhibitors, such as staurosporine and H-7, abolish MMP induction by growth factors [4,9]. These observations are also corroborated by this study, using Ro 31-8220, a more selective inhibitor of PKC, which does not inhibit cAMP, Ca^{2+}/calmodulin or receptor tyrosine kinases at the concentrations used here [55]. The transcription factor AP-1 is believed to be a major mediator of the effects of activated PKC, and there is some evidence that NF-κB is also involved [56]. The effects of Ro 31-8220 on gelatinase expression are therefore consistent with the proposed regulatory roles of AP-1 and NF-κB.

Divergent regulation of TIMP expression

We observed constitutive TIMP-1 and TIMP-2 expression in rabbit SMCs, which was not increased by either IL-1α or TGFβ alone, consistent with data for SMCs from other species [9,16]. Expression of TIMP-1 but not TIMP-2 was slightly increased by PDGF above the constitutive levels. This is consistent with data showing the presence of an active AP-1-binding site in the promotor region of the TIMP-1 gene [57]. This report is the first, to our knowledge, to describe the expression and regulation of TIMP-3 in SMCs. Expression of TIMP-3 was clearly different from that of TIMPs -1 and -2, in being induced by PDGF, TGFβ

and their combination. We have also shown that TIMP-3 is induced by serum (R. P. Fabunmi and A. C. Newby, unpublished work) with similar kinetics in rabbit SMCs to those reported in WI-38 fibroblasts [58]. Both TIMP-3 protein and mRNA were induced by PDGF or TGFβ alone. Synergistic effects were clearly visible on mRNA expression 4–12 h after stimulation, although synergism was not seen on protein expression after 24 h. The reasons for this are unknown but may be due to post-translational controls on TIMP-3 expression, which prevent the very high levels of mRNA induced by combinations of PDGF and TGFβ leading to greater protein secretion.

Our results clearly demonstrate a different response of 95 kDa gelatinase and TIMP expression to growth factors and cytokines. The presence of PDGF and IL-1α (perhaps part of an acute inflammatory response) would be expected greatly to increase 95 kDa gelatinase activity but only slightly to increase TIMP expression and hence favour net degradation of basement membrane and promote SMC migration and proliferation. Conversely, the presence of TGFβ would stimulate TIMP-3 expression, hence favouring basement-membrane formation and limiting SMC proliferation. Our results lead to the hypothesis that increased expression of TIMP-3 contributes to the self-limitation of SMC migration and proliferation and the net deposition of ECM components seen later after angioplasty.

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