Involvement of extracellular-matrix-degrading metalloproteinases in rabbit aortic smooth-muscle cell proliferation

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We investigated the influence of two structurally unrelated inhibitors of matrix-degrading metalloproteinases, Ro 31-4724 and Ro 31-7467, on the primary proliferation of smooth-muscle cells from rabbit aortic explants. Both agents inhibited proliferation in a concentration-dependent manner, but did not affect cell viability. Smooth-muscle cells grown out from explants secreted 95 kDa and 72 kDa gelatinase enzymes that were also inhibited in a concentration-dependent manner by Ro 31-4724 and Ro 31-7467. Interstitial collagenase and stromelysin were not detected. We conclude that metalloproteinases are likely to be involved in the initiation of smooth-muscle proliferation.

INTRODUCTION

Migration of smooth-muscle cells into the vascular intima and their subsequent proliferation are important events in the pathogenesis of atherosclerosis. Intimal smooth-muscle proliferation is also a primary cause of vessel occlusion after angioplasty, in the arteries of transplanted hearts and in saphenous-vein coronary-artery-bypass grafts [1]. There is therefore considerable interest in the underlying basis of intimal proliferation and in the development of inhibitors. Growth factors such as platelet-derived growth factor, basic fibroblast growth factor and insulin-like growth factor-1 are able to stimulate proliferation in collagenase-dispersed preparations of smooth-muscle cells [2]. In arteries *in vivo* and in full-thickness organ cultures of vessels *in vitro*, however, exposure to growth factors alone is insufficient to initiate proliferation [3–5]. This probably results from the inhibitory effect exerted by the normal extracellular matrix [6] which is removed during dispersion of cells by collagenase. These observations lead us to the hypothesis that both activation of growth-factor release and matrix remodelling are needed in concert to permit vascular smooth-muscle proliferation.

Matrix remodelling requires the action of proteinases, among which the neutral metalloproteinases (MMPs) appear to play a key role [7,8]. Several related MMPs have been described, including interstitial collagenase, which can cleave native fibrillar collagen at one specific locus per polypeptide chain. Gelatinases degrade denatured interstitial collagen and native basement-membrane collagens, whereas stromelysins have a wide specificity against matrix components, including gelatin, laminin, fibronectin, proteoglycan core proteins and elastin. These enzymes have been implicated in the tissue remodelling which accompanies inflammation, bone resorption, wound healing and the invasion of tumours, but their role in atherogenesis and other vascular pathologies has not been widely investigated. One report has described the production from calf aortic smooth-muscle cells of interstitial collagenase [9]. Endothelial cells are known to secrete gelatinases and the tissue inhibitor of metalloproteinases (TIMP) [10,11]. Macrophages and some smooth-muscle cells expressing stromelysin have been recently detected in human atherosclerotic plaques [12]. Although these observations provide some evidence for the secretion of MMPs by vascular cells, their role in proliferation has not been tested directly.

We therefore investigated the effect of two structurally dissimilar potent and specific synthetic MMP inhibitors [13] on the proliferation of smooth-muscle cells. We used a quantitative explant organ culture of rabbit aortic media [14,15], which allowed us to study the proliferation of cells that were initially interacting with their natural extracellular matrix. To clarify the nature of the targets for the inhibitors, we also investigated the production of MMPs by rabbit aortic smooth-muscle cells.

MATERIALS AND METHODS

The MMP inhibitors Ro 31-4724 and Ro 31-7467 (see Fig. 1 below) and purified 72 kDa gelatinase from human dermal fibroblasts were gifts from Roche Products, Welwyn Garden City, Herts., U.K. Stock solutions of Ro 31-4724 (20, 2.0 and 0.2 mM) and Ro 31-7467 (2.0, 0.2 and 0.02 mM) were prepared in 100% dimethyl sulphoxide (DMSO) and stored at 4 °C. Before use, each stock solution was diluted 200-fold in tissue-culture medium to give final concentrations of the agents in the range 0.1–100 µM and 0.5% DMSO. Sheep antisera to human 72 kDa gelatinase (X670) and pig 95 kDa gelatinase (A560) and partially purified rabbit interstitial collagenase and stromelysin were generously given by Dr. G. Murphy, Department of Cell and Molecular Biology, Strangeways Research Laboratories, Cambridge, U.K. Their preparation, and properties of the antisera, have been described previously [16]. Purified stromelysin-1, prostromelysin-1 and rabbit anti-(human recombinant stromelysin-1) serum were given by Dr. P. Davies, Merck, Rahway, NJ, U.S.A. Purified pig interstitial collagenase and rabbit anti-(pig collagenase) serum were generously given by Dr. T. Cawston, Rheumatology Research Unit, Addenbrooke's Hospital, Cambridge, U.K. Goat anti-(rabbit IgG)-alkaline phosphatase conjugate, 5-bromo-4-chloroindol-3-yl phosphate, ovalbumin and Nitroblue Tetrazolium were obtained from Sigma Chemical Co., Poole, Dorset, U.K. The source of the other reagents used has been also reported [15].

Preparation and culture of aortic medial explants

We used the method of McMurray *et al.* [14] as modified by Southgate & Newby [15]. Briefly, the thoracic aortas of healthy New Zealand White rabbits (age 9 weeks) were removed, opened out and the endothelium was removed by gentle abrasion. Strips

Abbreviations used: MMP, neutral (matrix) metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's modification of Eagle's medium.

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(1 cm long) of tunica media were peeled off the adventitia and were then cut up into 1 mm × 1 mm explants using a McIlwain tissue chopper in two planes at right angles. The explants were cultured individually in the wells of a 96-well microtitre plate, for the times indicated, in Dulbecco's modification of Eagle's medium (DMEM) containing 0.1 mM non-essential amino acids, 1 mM-sodium pyruvate, 8 mM-L-glutamine, penicillin/streptomycin (10 units/ml and 100 µg/ml respectively) and either 20% (v/v) foetal-calf serum or ovalbumin (1 mg/ml), as indicated. The culture medium was further supplemented with DMSO (0.5%) or the MMP inhibitors Ro 31-4724 (1–100 µM) or Ro 31-7467 (0.1–10 µM), with DMSO (0.5%) as indicated. For the time-course experiments the medium was removed after 3 days and replaced with fresh medium containing the inhibitors. Thereafter it was replaced every day. [6-³H]Thymidine (1 µCi/ml) was present in the media for 18 h before the cultures were terminated by the addition of ice-cold 10% (w/v) trichloroacetic acid. These acid extracts were used for measurements of ATP concentration [17] and [³H]thymidine incorporation by methods described previously [15].

Preparation of conditioned media and extracts of explants

To detect the secretion of MMP on day 3, serum-free conditioned medium was removed and pooled from 192 explants. The remaining explants were then extracted for 30 min at 4 °C with 0.1 ml of 50 mM-Tris/HCl (pH 8.0)/100 mM-NaCl/0.1% SDS and were then pooled. To produce larger quantities of pooled conditioned medium, approx. 500 explants from one aorta were distributed equally into five 75 cm² tissue-culture flasks and cultured in 20 ml of serum-containing medium, which was renewed every 3 days. On day 14, by which time cells covered approx. 70% of the substratum, the medium was removed and the cells were washed with two changes of Dulbecco's phosphate-buffered saline to remove serum and non-adherent cells. Cultures were then continued for up to 12 days in medium containing ovalbumin. The media were renewed and the conditioned medium was collected every 48 h. Conditioned medium and extracts of explants were centrifuged at 1500 g for 10 min to remove any cells and debris, and NaN₃ was then added to the supernatants to a final concentration of 0.1%. Supernatants were then concentrated 10-fold by ultrafiltration using Amicon Centriprep 10 concentrators (Amicon, Stonehouse, Gloucestershire, U.K.) and used immediately or stored at -20 °C until required.

Zymography

Gelatinase activity was measured in conditioned media subjected to electrophoresis into 7.5% (w/v) polyacrylamide gels containing 0.2% SDS [18] and of gelatin derived from rat tail collagen by the method of Heussen & Dowdle [19] (1 mg/ml). To measure stromelysin, β -casein (1 mg/ml) was used instead of gelatin [20] in 10% (w/v) polyacrylamide gels. Samples of concentrated conditioned media (10–20 µl) were mixed at room temperature (23 °C) with an equal volume of double-strength non-reducing sample buffer [18] and electrophoresed at 4 °C. After electrophoresis, SDS was removed from the gels by two washes for 15 min each with an aqueous solution of 2.5% (v/v) Triton X-100. The gels were then incubated at 37 °C in a buffer containing 50 mM-Tris/HCl (pH 8)/50 mM-NaCl/10 mM-CaCl₂/0.05% of Brij 35 for 18 h. To study inhibition of the enzymes, the buffer was supplemented with EDTA (20 mM), 1,10-phenanthroline (5 mM), phenylmethanesulphonyl fluoride (1 mM), *N*-ethylmaleimide (5 mM), pepstatin A (5 mM), Ro 31-4724 (0.01–100 µM) or Ro 31-7467 (1.0–50 µM) as indicated. The gels were then stained with aq. 0.1% Page Blue 83 (BDH, Poole,

Dorset, U.K.). Zones of lysis appeared as clear bands against the blue-stained background. The gels were calibrated with a high-molecular-mass standard mixture consisting of the following proteins: myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa) (Sigma). In addition, purified 72 kDa gelatinase, pro-stromelysin-I and active stromelysin-I were used as standards where appropriate.

Immunoprecipitation

Gelatinases were immunoprecipitated from 200 µl samples of 10-times-concentrated conditioned media by incubation with 10 µl of antiserum to 72 or 95 kDa gelatinase for 16 h at 4 °C. The mixture was then incubated with 50 µl of a 1:4 (v/v) suspension of Protein A-Sepharose (Pharmacia LKB Biotechnology, Milton Keynes, Bucks., U.K.) in 140 mM-NaCl/10 mM-sodium phosphate, pH 7.3 (PBS) for 2–4 h at 4 °C. Immune complexes were then precipitated by centrifugation at 13000 g for 5 min at 4 °C. The supernatant was removed and the pellet was washed five times with 50 mM-Tris/HCl (pH 8.3)/45 mM-NaCl/0.5% of Nonidet P40. Proteinases were released from the final pellet by the addition of 50 µl of double-strength SDS reducing buffer [18] and were then subjected to zymography as described above. Non-immune rabbit serum or unincubated culture medium were used as negative controls.

Western blotting

Samples of 50-times-concentrated conditioned media (20 µl) and pre-stained standard proteins (10 µl; Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.) were treated by boiling with an equal volume of double-strength reducing sample buffer and subjected to 10% (w/v) -PAGE [18]. Proteins were transferred electrophoretically at 100 V for 1 h using a buffer containing 25 mM-Tris, 192 mM-glycine, pH 8.3, and 20% (v/v) methanol (Bio-Rad Mini Trans-Blot apparatus; Bio-Rad Laboratories) to Schleicher and Schuell 0.45 µm-pore-size nitrocellulose membranes (Anderman Co., Kingston-Upon-Thames, Surrey, U.K.). The nitrocellulose was blocked by a 1 h incubation in 10% (w/v) skimmed-milk powder in PBS, pH 7.2, containing 0.1% (v/v) Tween 20 (PBST). After washing in PBST for 5 min, strips of nitrocellulose were incubated overnight at 4 °C with a 1:250 dilution of first antibody or non-immune rabbit serum (for controls) in PBS containing 0.05% Tween 20 and 1% (w/v) BSA (PBSTB). The next day the nitrocellulose strips were extensively washed with PBST and then incubated for 2 h at 4 °C in PBSTB containing a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibody. The strips were then extensively washed in PBST and then incubated for 5–20 min at room temperature with a chromogenic substrate mixture consisting of 8.25 mg of Nitroblue Tetrazolium, 4.25 mg of 5-bromo-4-chloroindol-3-yl β -D-phosphate dissolved in 25 ml of 0.1 M-NaCl/0.1 M-Tris/HCl (pH 9.5)/5 mM-MgCl₂. The sensitivity for detection of rabbit collagenase and stromelysin was determined by transferring to nitrocellulose membranes doubling dilutions of a partially purified preparation containing 11 µg/ml of each enzyme using a slot-blot apparatus. The membranes were then treated with specific antisera (or non-immune serum as a control) as described above. The limit of detection was 25 ng for collagenase and 100 ng for stromelysin.

RESULTS AND DISCUSSION

The structures of the two MMP inhibitors used in the present study are shown in Fig. 1. Ro 31-4724 is a hydroxamic acid derivative which has been shown to display similar potency against purified interstitial collagenase and gelatinase, but 3-fold

Fig. 1. Structures of MMP inhibitors

Fig. 2. Concentration responses of proliferation to inhibitors

Aortic explants ($n = 24$ for each condition) were cultured for 1 day ('Day 1 control') or 3 days in the presence of 0.5% DMSO (vehicle) or 0.5% of DMSO and the final concentrations of Ro 31-4724 or Ro 31-7467 indicated. In 3-day cultures, [^3H]thymidine (1 $\mu\text{Ci}/\text{ml}$) was present for the last 18 h. Explants were extracted with trichloroacetic acid and eight extracts were pooled to give three replicates for each condition. Incorporation of [^3H]thymidine (a) and ATP concentration (b) were measured as described in the Materials and methods section. Values were expressed as a percentage of those found in the explants cultured for 3 days in the presence of 0.5% DMSO. Values are means $\pm$ S.E.M. for three or four separate experiments each performed in triplicate. *Significantly ($P < 0.05$) less than 100%.

Table 1. Effect of MMP inhibitors on the outgrowth of smooth-muscle cells

The percentage coverage of the substratum outside the explants was scored visually in 24 wells for each condition after 3 and 7 days in culture in the presence of 0.5% DMSO (vehicle) without or with the MMP inhibitors shown. Values are means $\pm$ S.E.M. for three separate experiments. Statistical significance: * $P < 0.05$ versus vehicle control on the corresponding day.

Condition	Time (days)	Visually observed coverage		
		None	< 5 %	5–50 %
Vehicle control	3	24	0	0
	7	3 $\pm$ 1	6 $\pm$ 5	15 $\pm$ 3
Ro 31-4724 (100 μM)	3	24	0	0
	7	12 $\pm$ 2*	8 $\pm$ 3	4 $\pm$ 2*
Ro 31-7467 (10 μM)	3	24	0	0
	7	8 $\pm$ 2	6 $\pm$ 1	10 $\pm$ 3

Culture of explants with Ro 31-4724 or Ro 31-7467 for 3 days inhibited proliferation in a concentration-dependent manner (Fig. 2a). Approx. 50% inhibition occurred with 10 μM -Ro 31-4724 or -Ro 31-7467 and was still incomplete with 100 μM -Ro 31-4724 (Fig. 2a); it was not possible to use 100 μM -Ro 31-7467 owing to its poor solubility. On day 3 there was no visible outgrowth of cells from the explants in either the presence or absence of inhibitors (Table 1). The ATP concentration, which we have shown to be a marker of biomass and cell viability in this tissue [15], increased between day 1 and day 3 in culture (Fig. 2b). Neither MMP inhibitor reduced the concentration of ATP to below the value obtained on day 1 (Fig. 2b) and therefore did not appear to show cytotoxicity. The increase in ATP concentration between days 1 and 3 was, however, significantly attenuated by 100 μM -Ro 31-4724, consistent with its effect to inhibit proliferation. Because serum is known to contain inhibitors of MMP, a second series of experiments was carried out in serum-free medium containing 1 mg of ovalbumin/ml. We took advantage of the initial partial serum-independence of proliferation under these conditions. Thymidine incorporation (d.p.m./ μg of DNA) increased from 400 ± 100 on day 1 to 42000 ± 9000 on day 3 in the absence of serum compared with 124000 ± 11000 in the presence of 20% serum ($n = 7$). Thymidine incorporation on day 3 in the absence of serum was not affected by the vehicle DMSO ($85 \pm 12\%$), but was significantly ($P < 0.05$) decreased by 10 μM -Ro 31-4724 to $66 \pm 6\%$, by 100 μM -Ro 31-4724 to $16 \pm 5\%$ and by 10 μM -Ro 31-7467 to $56 \pm 12\%$ ($n = 3$). Thus the effect of the MMP inhibitors on proliferation was similar in the presence and absence of serum (see Fig. 2a).

In a third series of experiments, the effects on proliferation of the two MMP inhibitors was measured in serum-containing medium at different time points. As we have previously demonstrated using this model [15], the growth curve (Fig. 3a) shows three components, namely a 2-day lag, a rapid but unsustained first phase of proliferation before cells emerge from the explants (days 2–4), and a second phase of rapid proliferation accompanied by outgrowth of cells (Table 1). There was significant incorporation of [^3H]thymidine for the first time on day 3 in either the presence or absence of MMP inhibitors (Fig. 3a). This suggests that, within the resolving power of these experiments, neither inhibitor extended the lag for proliferation. The time-course experiments confirmed that both agents significantly inhibited the first phase of [^3H]thymidine incorporation on day 3 (Fig. 3a). The increase in ATP concentrations between days 1 and 3 was similar in the presence and absence of the MMP

lower potency against stromelysin [13]. Ro 31-7467 is a phosphinic acid analogue with 2-fold lower potency against interstitial collagenase than Ro 31-4724 [13]. Ro 31-7467 also displays 30-fold lower potency against gelatinase and a 20-fold lower potency against stromelysin compared with Ro 31-4724 [13].

Fig. 3. Time course of the effect of MMP inhibitors on proliferation

Aortic explants ($n = 24$ for each time point) were incubated for the times shown with 0.5% of DMSO (vehicle, $\square$) or 0.5% of DMSO and 100 μM -Ro 31-4724 ($\bullet$) or 10 μM -Ro 31-7467 ($\blacksquare$); $[^3\text{H}]$ -thymidine (1 $\mu\text{Ci}/\text{ml}$) was added for the last 18 h. Explants were extracted with trichloroacetic acid (see the legend to Fig. 2) and incorporation of $[^3\text{H}]$ thymidine (a) and ATP concentration (b) were measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. (when larger than the symbol) from three separate experiments, each performed in triplicate. Statistical significance: * $P < 0.05$ versus 0.5% of DMSO on the same day.

inhibitors, although it tended to be decreased by 100 μM -Ro 31-4724 (Fig. 3b). Both MMP inhibitors also inhibited the second phase of $[^3\text{H}]$ thymidine incorporation (Fig. 3a) without significantly affecting ATP concentrations (Fig. 3b). Inhibition during the second phase was more pronounced in the case of 100 μM -Ro 31-4724, which significantly inhibited outgrowth of cells (Table 1). Thus the time-course studies demonstrated that MMP inhibitors affected the proliferation and migration of cells within the explants. An additional effect on the cells that migrated out cannot, however, be excluded.

Release of MMPs from cultured cells

The primary cells which grew out from the explants in medium containing serum stained uniformly with a monoclonal antibody to smooth-muscle cell actin (results not shown), which confirmed their identity and homogeneity. To clarify the mechanism underlying inhibition of proliferation, we investigated whether these cells produce MMPs sensitive to Ro 31-4724 and Ro 31-7467. We studied serum-free conditioned media to avoid interference from serum proteins.

Gelatinase

When conditioned media obtained after the first 3 days in culture were subjected to zymography in gels impregnated with gelatin, major zones of lysis were observed consistently with apparent molecular masses of approx. 95, 72 and 68 kDa (Fig.

Fig. 4. Detection of MMP activity using zymography

Rabbit aortic explants from four different rabbits were grown in serum-free medium for 3 days, after which the medium was removed and the remaining explants were also extracted. Conditioned media and extracts were then concentrated 10-fold. Zymography was then performed using gels impregnated with gelatin as described in the Materials and methods section. Samples (15 μl) were applied as follows: lane 1, unincubated culture medium; 2, molecular-mass (M) markers; 3-6, different conditioned media; 7-10, corresponding extracts of explants; 11, an example of conditioned medium collected after 14 days as described in the legend to Fig. 5.

4). Extracts of the explants themselves showed a similar, although much weaker, pattern (Fig. 4), indicating that the enzymes were predominantly secreted into the medium. Bands of the same molecular mass were obtained in conditioned medium obtained after more prolonged culture, although there was some variability in the relative proportion of the 95 kDa and 72 kDa bands from preparation to preparation (compare Figs. 4, 5 and 6). Nevertheless, when media were obtained repeatedly at 2-day intervals from the same culture, the molecular masses and intensities of the zones of lysis appeared to be similar for all the time points (Fig. 5a), which suggested that the enzymes responsible were continuously released. These were therefore characterized from pooled conditioned media. The zones of lysis were abolished by 1,10-phenanthroline (results not shown) or EDTA (Fig. 5b), but phenylmethanesulphonyl fluoride, *N*-ethylmaleimide and pepstatin A failed to cause any detectable inhibition (Fig. 5b), thereby establishing that they were the result of MMPs. Antiserum to the human fibroblast 72 kDa gelatinase precipitated the enzymes in the conditioned media that gave rise to the 72 and 68 kDa bands of lysis, and antiserum to the pig 95 kDa gelatinase precipitated the 95 kDa band (Fig. 5c). Samples of conditioned medium after treatment with a combination of both antisera showed no bands of lysis in zymograms (results not shown). Thus rabbit aortic smooth-muscle cells secreted MMPs that were closely related immunologically to those that have been well characterized in other cells [7,8]. From the data on other cells [7,8] the zymograms appeared to reveal the presence of a 72 kDa progelatinase, the corresponding 68 kDa active enzyme, and a distinct 95 kDa gelatinase.

Stromelysin and interstitial collagenase

No zones of lysis were detected when gels impregnated with casein (a preferred substrate for stromelysin) rather than gelatin were used, even after prolonged incubation (results not shown). Furthermore, we were unable to detect the presence of stromelysin in the conditioned medium by Western blotting (Fig. 6). We were similarly unable to detect interstitial collagenase in a functional assay (results not shown) or by Western blotting (Fig. 6).

Effects of Ro 31-4724 and Ro 31-7467 on gelatinase activity

The gelatinase activities detected by zymography were inhibited in a concentration-dependent manner by Ro 31-4724 (Fig. 7a) or Ro 31-7467 (Fig. 7b). Although this method of measuring enzyme activity is not strictly quantitative, the results

Fig. 5. Characteristics of MMP activity

Rabbit aortic explants were grown in serum containing medium for 14 days and then transferred to serum-free culture. The medium was replaced every 2 days and the conditioned media were then concentrated 10-fold. Zymography was performed using gels impregnated with gelatin as described in the Materials and methods section. Each panel shows a representative of three similar experiments. (a) Conditioned media were collected at different intervals from the same cultures and 10 μ l samples were subjected to zymography as follows: lane 1, high-molecular-mass (*M*) markers; 2, purified 72 kDa gelatinase standard; 3 and 4, 0–2 days; 5 and 6, 2–4 days; 7, 4–6 days; 8, 6–8 days; 9, 8–10 days; 10, 11–12 days. (b) Serum-free conditioned media from the various time intervals were pooled, and 10 μ l samples were subjected to electrophoresis in gels containing gelatin. Strips of gel were then developed in buffer containing 0.5% DMSO vehicle and the following additions: lane 1, high-molecular-mass markers; 2, vehicle alone; 3, 5 mM-*N*-ethylmaleimide; 4, 1 mM-phenylmethanesulphonyl fluoride; 5, 5 mM-pepstatin A; 6, 20 mM-EDTA. (c) Aliquots of the same pooled media used in (b) were subjected to immunoprecipitation with antibodies to 72 kDa and 95 kDa gelatinases as described in the Materials and methods section. The immunoprecipitates were solu-

suggested that the potency of Ro 31-4724 was somewhat greater than that of Ro 31-7467, consistent with their reported potencies against purified gelatinase [13]. Both agents appeared to be more potent against the 95 kDa than against the 72 kDa enzyme, which has not been noted previously. The bands of lysis were almost abolished by 10 μ M-Ro 31-4724 (Fig. 7a) or 50 μ M-Ro 31-7467 (Fig. 7b).

Role of MMPs in smooth-muscle proliferation

We have shown that two inhibitors of MMPs, Ro 31-4724 and Ro 31-7467, inhibited the proliferation of rabbit aortic smooth-muscle cells from explants. The inhibition was most pronounced in the first 3 days of culture, which implies that MMPs were involved in remodelling the native extracellular matrix within the explants. MMPs also appeared to be involved in permitting migration of cells from the explants. The fact that the inhibitors were structurally unrelated reinforces the conclusion that inhibition of MMP activity was responsible for the effects on proliferation and migration. An alternative possibility, namely cytotoxicity, was unlikely to account for inhibition of proliferation because ATP concentrations were not reduced below the initial values. Indeed, the effects of Ro 31-4724 on ATP concentration were closely similar to those of other inhibitors of proliferation, such as 8-bromo cyclic AMP, that have been characterized with this model [15].

We also demonstrated that rabbit aortic smooth-muscle cells secreted 72 kDa and 95 kDa gelatinases, which were sensitive to both MMP inhibitors. Although it was not practical to study MMP activity in serum-containing medium, the pattern of secretion was very similar in the first 2 days and up to 12 days after serum removal, which argues against a major effect of serum on MMP secretion. Inhibition of proliferation by MMP inhibitors was also observed in either serum-containing or serum-free medium. Similar concentrations of the agents were found to inhibit proliferation and gelatinase activity in the zymogram assay. The apparent potency of the agents is lower than the sub-micromolar values that have been reported using synthetic peptide substrates, but similar to those found to inhibit matrix degradation in tissue-culture experiments [13]. The agents are competitive inhibitors [13], and thus the unknown effective substrate concentration within the explants will influence their potency. Instability over the relatively long time course of the culture and zymogram experiments may also have reduced their potency.

The most direct interpretation of our results is that gelatinase activity is necessary for vascular smooth-muscle proliferation. Arterial smooth-muscle cells are known to be surrounded by a pronounced pericellular basement membrane in which type IV collagen is a major constituent [21], and proliferation of the cells is normally suppressed by inhibitory components of the extracellular matrix [6]. Our observations may be explained if basement-membrane degradation by gelatinases permits migration and subsequent proliferation of the cells. Given the broad specificity of the inhibitors for MMPs, we cannot rule out the involvement of interstitial collagenase and/or stromelysin. We were not, however, able to detect the presence of these enzymes. Although reproducible in ten separate experiments, the inhibition of proliferation by the agents used was incomplete. This may signify that proteinases other than MMPs are also involved in

bilized and subjected to zymography after as follows: lane 1, high-molecular-mass markers; 2, without immunoprecipitation; 3, with non-immune rabbit serum; 4, with anti-(72 kDa gelatinase) antiserum; 5, with anti-(95 kDa gelatinase) antiserum.

Fig. 6. Detection of collagenase and stromelysin by Western blotting

Samples (20 μ l) of unincubated culture medium, pooled 50-fold-concentrated conditioned media and standard proteins were subjected to electrophoresis under reducing conditions into 10% (w/v) polyacrylamide gels, and the separated proteins were transferred to nitrocellulose filters as described in the Materials and methods section. Filters were developed with antisera to stromelysin (lanes 2–4) or interstitial collagenase (lanes 5–7) as detailed in the Materials and methods section. The samples were as follows: lane 1, pre-stained low-molecular weight (*M*) markers; 2, unincubated medium; 3, conditioned medium; 4, 400 ng of stromelysin-1; 5, unincubated medium; 6, conditioned medium; 7, 280 ng of interstitial collagenase.

Fig. 7. Effect of MMP inhibitors on gelatinase activity

Aliquots (10 μ l) of pooled concentrated conditioned media were subjected to electrophoresis in gels containing gelatin, and strips were developed by incubating in buffer containing 0.5% DMSO (vehicle) and MMP inhibitors as follows: (a) Lane 1, high-molecular-mass (*M*) markers; 2, vehicle alone; 3, 0.1 μ M-Ro 31-4724; 4, 0.5 μ M-Ro 31-4724; 5, 1 μ M-Ro 31-4724; 6, 10 μ M-Ro 31-4724; 7, 100 μ M-Ro 31-4724. (b) Lane 1, high-molecular-mass markers; 2, vehicle alone; 3, 1 μ M-Ro 31-7467; 4, 10 μ M-Ro 31-7467; 5, 50 μ M-Ro 31-7467. The figures shown are representative of three similar experiments.

matrix remodelling in rabbit aorta. Indeed, recent data strongly point to a role for plasminogen activators in this context [22].

There is at present little information on the secretion of MMPs

by vascular smooth-muscle cells. One previous study detected interstitial collagenase in bovine aortic smooth-muscle cells, although it was present as a complex with TIMP, its endogenous inhibitor [9]. The presence of TIMP would not, however, have interfered with the detection of collagenase by Western blotting, and so is not the reason for our failure to detect the enzyme. Renal mesangial cells, which are closely related to vascular smooth-muscle cells, have been more extensively studied. They are known to secrete 72 kDa gelatinase constitutively [23,24] and to up-regulate the secretion of 95 kDa gelatinase in response to cytokines [25]. Secretion of interstitial collagenase and stromelysin appears to be less important, although mRNAs for both enzymes have been detected [25]. These data suggest that the pattern of MMP expression in vascular smooth-muscle cells and mesangial cells may be similar, although further work will be needed to confirm this.

It is generally agreed that growth-factor production is the primary initiator of smooth-muscle-cell proliferation during vascular repair and in vascular diseases. Nevertheless, a number of experiments conducted *in vivo* and *in vitro* have demonstrated the need for additional factors. In particular a requirement for cell injury has been noted in several models [4,5,26]. The present paper identifies activation of MMPs, particularly gelatinase, as one ancillary factor that might account for these observations.

The pathogenesis of intimal smooth-muscle proliferation in atherosclerosis, in vein grafts and after angioplasty probably involves growth factors from endothelium and inflammatory cells as well as smooth-muscle cells [27]. Likewise, MMP activity might be derived from any of these cells, all of which have now been shown to secrete a spectrum of these proteinases. Whatever the origin of MMPs, selective inhibitors of the enzymes may have a role in arresting atherosclerosis. They may also be effective against reocclusion of arteries after angioplasty and in preventing occlusion in the transplanted heart and in vein grafts.

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