Surgical preparative injury and neointima formation increase MMP-9 expression and MMP-2 activation in human saphenous vein

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Abstract

Objectives: Injury stimulates smooth muscle cell (SMC) migration and proliferation by mechanisms that are incompletely understood. Surgical preparative injury is an important determinant of neointimal thickening in human saphenous vein bypass grafts. We investigate here whether basement-membrane-degrading metalloproteinases (MMPs) are stimulated by surgical preparation and culturing of human saphenous veins in organ culture. Methods: Secretion of MMP-2 and MMP-9 was measured by zymography and Western blotting. Sites of MMP secretion were localised by immunocytochemistry and in situ hybridisation. Results: Freshly isolated veins secreted pro-MMP-2 and much lower amounts of active MMP-2 and pro-MMP-9. MMP-2 was expressed in all cells whereas MMP-9 expression was confined to endothelial cells and at low levels to 5 ± 10% of medial SMC. Surgical preparative injury increased pro-MMP-2, active MMP-2 and pro-MMP-9 secretion. MMP-9 expression 3 h after surgical preparation occurred at high levels in 89 ± 3% of medial SMC (P < 0.05 vs. freshly isolated, n = 6). Culturing in serum for 12 days increased pro-MMP-2, active MMP-2 and pro-MMP-9 secretion to equal levels in freshly isolated and surgically prepared veins. MMP-9 expression was greatest in the highly proliferative neointimal SMC and was more widespread in medial SMC of surgically prepared than freshly isolated veins (89 ± 3 vs. 67 ± 11%; P < 0.05, n = 6). Conclusions: The data provide new insights into the mechanisms underlying human SMC proliferation. Activation of MMP-2 and increased MMP-9 expression are shown to be important components of the response to injury in this model. Furthermore, MMP-9 expression is closely associated with medial and neointimal SMC proliferation.

Keywords: Vascular smooth muscle; Arteriosclerosis; Coronary artery bypass grafting; Metalloproteinases; Smooth muscle cell proliferation; Human, veins

1. Introduction

Neointima formation is a characteristic feature of atherosclerosis formation, restenosis after angioplasty and the adaptation of autologous human saphenous vein to implantation as coronary artery bypass grafts. Injury is thought to be an important stimulus to neointima formation mediated in part by growth factors, reviewed in Refs. [1,2]. For example, we have demonstrated directly the involvement of platelet-derived growth factor (PDGF) expression in neointima formation in pig vein grafts [3] and organ cultures of human saphenous vein [4]. However, the effects of injury are not entirely explained by growth factors because injury stimulates medial smooth muscle cell (SMC) proliferation in organ cultures even in the presence of serum [5–7]. Similar in vivo data in the rat carotid angioplasty model show that injury stimulates neointima formation particularly when there is a sufficient source of endogenous or infused growth factors [7–12]. These effects may be explained by the ability of injury to stimulate degradation of the basement membrane surrounding SMC, thereby facilitating their migration and proliferation [13]. The involvement of basement-membrane-degrading matrix metalloproteinases (MMPs) in SMC migration and proliferation in vitro has been demonstrated directly using MMP...
inhibitors [13]. A similar role for MMPs and plasminogen activators has been established after balloon angioplasty in the rat carotid artery [14–19] and in transgenic mice [20].

However, little is known about the role of matrix-degrading enzymes in humans. The active forms of stromelysin (MMP-3) and MMP-9 have been detected in aneurysmal aortic wall [21–23] and with interstitial collagenase (MMP-1) in the shoulders and foam-cell-rich regions of atherosclerotic plaques [24–27]. These observations at the late stages of vascular disease, when the rates of SMC migration and proliferation are thought to be low [28], do not yield a clear picture of the pathophysiologic function of MMPs which may relate to medial thinning and plaque instability rather than neointima formation [25,26].

To relate the expression of the basement-membrane-degrading MMP-2 (gelatinases A) and MMP-9 (gelatinase B) to the response to injury and neointima formation in human tissue, we used the well-established human saphenous vein organ model [6,29]. As in grafted veins, a viable neointima is formed, mediated by SMC migration and proliferation [6]. The stimulus for this in organ cultures is serum which mimics the effect of activated platelets in vivo [6]. A similar response is observed in human internal mammary arteries [30]. MMP activity secreted into conditioned medium was compared by zymography and Western blotting in veins before and after surgical preparation and culture. MMP-2 and MMP-9 mRNA and protein expression were localized by in situ hybridization and immunocytochemistry, respectively.

2. Methods

2.1. Materials

All reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK) except for those listed below. EM-1 and LM-1 emulsion, [3H]thymidine and α-35S [UTP] were obtained from Amersham International, sodium heparin from CP Pharmaceuticals, L-glutamine, penicillin and streptomycin from Gibco BRL, RPMI 1640 culture media and amphotericin B from ICN Flow, foetal calf serum (FCS) from Imperial Laboratories, papaverine hydrochloride from McCarthy Medical and gentamicin from Roussel. QB-end-10 monoclonal antibody was obtained from Oxoid Unipath Ltd, the in vitro transcription kit and PCR mini preps from Promega and HT1080 cells derived from a human fibrosarcoma from European Collection of Animal Cell Cultures. Partial cDNA sequences for human MMP-2 in the pUC19 vector and human MMP-9 in the pBlueScript KS vector (PBS 92174) were generous gifts from Professor Tryggvason (University of Oulu) and Dr. Goldberg (Washington University School of Medicine), respectively. Sheep polyclonal antibodies to MMP-2 (X670/10) and MMP-9 (A560/8), prepared as described previously [31], were generous gifts of Dr. G. Murphy (Strangeways Research Laboratory, Cambridge, UK). Purified human MMP-2 was obtained from TCS and MMP-9 as a generous gift from Dr. G. Murphy (Strangeways Research Laboratory, Cambridge, UK).

2.2. Vein collection

Human saphenous vein segments were obtained and cultured by a modification of the method of Pederson and Bowyer [32] as described previously by Soyombo et al. [29]. Human saphenous vein segments were obtained from 12 patients undergoing coronary artery bypass grafting. Briefly, ‘freshly isolated’ segments that had been subjected to minimal dissection were obtained from the ankle as soon as practical after the first incision. ‘Surgically prepared’ segments were obtained from the ankle region of each of the same patients after the completion of the last proximal anastomosis (at most 3 h after initial dissection). These veins had been subjected to adventitial stripping, side branch ligation, gentle manual distension and storage in heparinized blood. Ten patients were male and 2 female and the mean age of the patients was 58.1 years (range 40–74 years). Ethical permission was obtained from the United Bristol Hospital Trust ethics committee (Ref. E2847). Vein segments were collected in sterile 20 mmol/l Hepes-buffered RPMI 1640 tissue culture media containing 0.225 mg/ml papaverine hydrochloride as a vasorelaxant, 5 µg/ml amphotericin B and 20 IU/ml sodium heparin.

2.3. Organ culture

Segments were placed in wash medium consisting of 20 mmol/l Hepes-buffered RPMI 1640 tissue culture media supplemented with 2 mmol/l L-glutamine, 8 µg/ml gentamicin, 100 IU/ml penicillin and 100 µg/ml streptomycin. The adventitia was removed and then the vein cut longitudinally, pinned out onto set Sylgard resin with minute pins and divided into 6 replicate 5–10 mm segments. One segment was fixed in 10% formal buffered saline and another stored in liquid nitrogen for ATP analysis. The 4 remaining segments were pinned down with minute pins endothelial surface uppermost on polyester mesh resting on Sylgard resin in glass Petri dishes. One of these segments was placed in serum-free incubation media (2 g/l bicarbonate-buffered RPMI 1640 tissue culture media supplemented with 2 mmol/l L-glutamine, 8 µg/ml gentamicin, 100 IU/ml penicillin, 100 µg/ml streptomycin) for 48 h at 37°C under 95% air/5% CO2, after which conditioned medium was collected. Two of the remaining segments were cultured for 12 days in incubating medium supplemented with 30% (v/v) FCS at 37°C under 95% air/5% CO2, changing the media every 2 days and then placed in serum-free incubation medium for 48 h, after which conditioned medium was again collected. To
determine cell proliferation, the final segment was cultured for 14 days in incubating medium supplemented with 30% (v/v) FCS and 1 µCi/ml [3H]thymidine at 37°C under 95% air/5% CO2, changing the media every 2 days. Vein segments from which conditioned medium was removed were then washed twice with phosphate-buffered saline (PBS, 0.15 mol/l NaCl, 7.5 mmol/l Na2HPO4·12H2O, 1.9 mmol/l NaH2PO4·2H2O, pH 7.4). One of the segments collected at day 14 was frozen in liquid nitrogen for ATP analysis and the remaining segments were fixed in 10% buffered formal saline, processed and paraffin wax-embedded. Blocks of embedded tissue were oriented at right angles to the original direction of blood flow, transverse 5 µm thick sections were then cut and mounted on 3-aminopropyl-triethoxysilane-coated slides. The conditioned medium was immediately frozen and stored at −20°C with 0.01% sodium azide until analysis.

2.4. Histology

Serial sections were examined by Miller’s elastic van Gieson and Harris’ haematoxylin and eosiin staining [33]. Vascular SMC were identified by immunocytochemistry using monoclonal anti-α-smooth-muscle-actin antibody (clone 1A4) as described by [29]. Endothelial cells were identified by immunocytochemistry using QB-end-10 antibody as described [30]. Proliferating cells were identified in the vein segments cultured in the presence of 1 µCi/ml [3H]thymidine by autoradiography as described previously [4,6].

2.5. Zymography

Gelatinase activity in conditioned media collected from 12 paired organ cultures between days 0 and 2 and days 12 and 14 (n = 12) and from HT1080 cells (positive control) was measured by zymography. Conditioned media collected from one patient were always compared on the same gel. To correct for variations in vein segment size, all conditioned media were diluted to the equivalent of 20 mg wet weight of vein per 5 ml of conditioned medium with serum-free incubation media. Conditioned media were subjected to electrophoresis on 7.5% (w/v) PAGE gel containing 0.2% (w/v) SDS [34] supplemented with 2 mg/ml gelatin [35], as described previously [13]. All gels were calibrated with a high-molecular-mass protein standard mixture containing 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). In addition, for standardization, on each gel an aliquot of a standard conditioned medium collected from HT1080 cells (which secrete both MMP-2 and 9) was run. The mean gelatinase activities (n = 12) were estimated relative to the HT1080 standard by densitometric scanning of the zymograms as described below.

For further identification, immunoprecipitation of gelatinase isoforms was conducted using purified immuno-globulins from anti-(human gelatinase A), anti-(pig gelatinase B) or non-immune sheep sera. Aliquots of conditioned media (200 µl) were incubated with 10 µl of 1 M Tris/HCl, pH 7.2 and 50 or 100 µg of immunoglobulin for 60 min at 37°C; 50 µl of a 1:4 (v/v) suspension of protein G sepharose in phosphate-buffered saline (PBS) was added and the mixture was incubated with shaking for 2 h at 4°C. The suspension was centrifuged at 1000 × g for 2 min and aliquots of the supernatant (10 µl) were then removed and subjected to zymography.

2.6. Western blotting

Anti-MMP-2 and anti-MMP-9 antisera were produced in rabbits with synthetic peptides synthesized to the dissimilar C-termini of the enzymes. The sequences were CLKSVKFGSIKSDWLG and KQVGYVTYDILQCPED for MMP-2 and MMP-9, respectively. These peptides were coupled to key hole limpet haemocyanin and then injected into New Zealand white rabbits. The antisera generated were characterized using slot and Western blotting of conditioned medium from HT1080 cells, which express both MMP-2 and MMP-9, and with authentic purified human standards. Slot blotting with serial 10-fold dilutions of antisera established that 1:1000 dilutions gave maximal detection. Western blotting of purified proteins demonstrated that the antisera for one gelatinase exhibited no cross-reactivity with the other.

Conditioned medium collected from organ cultures (n = 6) was concentrated 50-fold using Amicon 30 centrifugal concentrators. Sample proteins were resolved using 5% (w/v) stacking and 8% (w/v) resolving PAGE gels. MMP-2 and MMP-9 proteins were detected by 1:1000 dilution of rabbit anti-human MMP-2 and MMP-9 antisera, 1:2000 dilution of donkey anti-rabbit IgG hors eradish peroxidase conjugated antibody and ECL chemiluminescent detection system. To demonstrate the specificity of the detection system, the antisera was pre-absorbed with 10 µg/ml of the corresponding peptide.

2.7. Densitometric scanning

MMP-2 and MMP-9 protein detected by zymography and Western blotting were quantified by densitometric scanning of gels and autoradiographs, respectively, using a Bio-Rad GS-690 imaging densitometer. The results were expressed as O.D. × mm2 and the means calculated. To correct for variation between zymograms and Western blots, the O.D. per mm2 results were expressed as a percentage of the standard HT1080 sample.

2.8. Preparation and validation of 35S-labelled ribonucleic acid (RNA) probes

Antisense and sense MMP-2 and MMP-9 35S-labelled RNA probes of 507 bp and 593 bp were generated by the
polymerase chain reaction (PCR) from cDNA sequences as described previously [36]. Briefly, PCR products were cloned into the PCR II TA cloning vector which contains flanking Sp6 and T7 transcription sites. Orientation of the inserted PCR product within the vector was assessed by both diagnostic restriction enzyme digestion and DNA sequence analysis using M13 forward and reverse primers. To produce antisense probes clones were digested with SpeI (MMP-2) or EcoRV (MMP-9) and transcribed in the presence of α-35S[UTP] (specific activity > 1000 Ci/mmoll) using T7 (MMP-2) or Sp6 (MMP-9) RNA polymerase and an in vitro transcription kit as specified in the manufacturer’s instructions. To produce the sense probes, the clones were digested with EcoRV (MMP-2) and SpeI (MMP-9) and transcribed using Sp6 (MMP-2) and T7 (MMP-9) as above.

The specificity of the probes was established by dot blot hybridization against serial 10-fold dilutions of the plasmids used as templates. As expected, both antisense and sense probes detected the same quantity of the double-stranded parent vector, but there was no detectable hybridization to the same quantity of the vector for the other MMP (data not shown). Furthermore, the MMP-2 chain antisense probes detected mRNAs of 3.1 kb and the MMP-9 chain antisense probes detected mRNA of 2.8 kb in Northern blots of extracts of HT1080 cells, which correspond to the known sizes of the cognate mRNAs [36].

2.9. In situ hybridization

In situ hybridizations with antisense 35S-labelled RNA probes were carried out by a modification of the method of Angerer and Angerer [37] as described previously [4]. After development, the sections were lightly counterstained with haematoxylin. Control slides were treated in the same manner and either pre-hybridized for 2 h with 10-fold molar excess of unlabelled RNA probe in hybridization buffer or hybridized with 35S-labelled sense RNA probes. A positive control section of HT1080 cells, which are known to express MMP-2 and MMP-9 mRNA, was always included.

2.10. Immunocytochemistry for MMP-2 and MMP-9

Cells expressing MMP-2 and MMP-9 protein were identified in paraffin sections by immunocytochemistry as described previously [4] using the primary rabbit anti-human MMP-2 and MMP-9 antiseras (diluted 1:1000) described above. A negative control where the antibody was pre-absorbed with 10 μg/ml of the appropriate synthetic peptide and a positive control of paraffin-embedded HT1080 cells were always included. Cells expressing MMP-2 and MMP-9 protein were stained brown and nuclei stained blue whilst negative cells had blue nuclei and no staining in the cytoplasm.

2.11. Statistical analysis

Data were analysed using Student’s t-test. Statistical significance was accepted when P < 0.05.

3. Results

3.1. Histological and biochemical changes during surgical preparation and organ culture

The characteristics of the response of human saphenous vein to surgical preparation and organ culture have been extensively documented previously [4,6,27,38]. We confirmed that the freshly isolated vein segments used here exhibited typical saphenous vein histology with an almost complete endothelial coverage before and after 14 days in organ culture (results not shown). Surgical preparation led to injury to the vessel wall including partial removal of the endothelium and damage to the medial layer, as evidenced by a fall in ATP concentration from 227 ± 20 nmol/g wet wt. in freshly isolated to 133 ± 26 nmol/g wet wt. in surgically prepared veins (mean ± s.e.m., n = 6, P < 0.05). After culture, a highly cellular, elastin-poor neointima developed in both freshly isolated and surgically prepared vein segments. The neointimal cells were identified as SMC beneath a single layer of endothelial cells (results not shown). The ATP concentration in surgically prepared vein partially recovered after 14 days in culture to 165 ± 25 nmol/g wet wt. (n = 6, P < 0.05), as also demonstrated previously [6,27,38]. Although the neointima formed in the freshly isolated and surgically prepared veins appeared histologically similar, the neointima was significantly thicker in the surgically prepared veins (32 ± 4 vs. 51 ± 8, n = 6, P < 0.05). The percentage of proliferating medial SMC detected by autoradiography was significantly greater in surgically prepared than freshly isolated vein segments after 14 days in culture (18 ± 3 vs. 8 ± 4 cells per mm2, n = 6, P < 0.05), confirming that surgical preparation stimulated medial SMC proliferation [6,38].

3.2. Measurement of gelatinolytic activity by zymography

The secretion of MMP-2 and MMP-9 into conditioned media of organ cultures was investigated first by gelatin zymography and densitometric scanning. Fig. 1A shows a representative zymogram (n = 12) with 3 major zones of gelatinase activity of 68, 72 and 95 kDa molecular weights (Fig. 1). The appearance of these activities was abolished when vein segments were cultured in the presence of 0.1 mmol/1 cycloheximide for 48 h (results not shown), which indicates that the gelatinase activity arose from new synthesis and secretion. Assignments of the bands in these human derived media were validated by comparison with authentic standards and by immunoprecipitation with specific antibodies (Fig. 1B). The 95 kDa band co-migrated
with authentic human progelatinase B and an antiserum to pig gelatinase B selectively depleted the 95 kDa band. An antibody to human gelatinase A selectively depleted the 72 and 68 kDa bands but not the 95 kDa band. These data confirm previous studies using rabbit aortic tissue, in which a similar 95 kDa band was identified as the pro-form of MMP-9 and the 68 kDa and 72 kDa bands as the active and pro-form of MMP-2 [13].

Quantitative analysis of the gelatinase activity detected by zymography was carried out using densitometric scanning. When increasing volumes of the same conditioned medium were subjected to zymography and densitometric analysis, a linear response was obtained followed by a plateau phase (results not shown). All zymograms were loaded to yield bands within the linear range of measurement. In addition, to control for possible variation in gelatin concentration, staining and destaining the same medium after immunoprecipitation with anti-gelatinase B anti-B, anti-gelatinase A (anti-A) or non-immune IgG (NI) and to authentic human progelatinase B (pro-GB).

Furthermore, after culture of either vein type in serum for 12 days, secretion of each of the 68, 72, and 95 kDa bands over a further 48 h in serum-free medium was significantly higher than from freshly-isolated vein over the first 48 h (Fig. 1 and Table 1). After culture, there was no difference in any value between freshly isolated and surgically prepared veins.

### 3.3. Measurement of MMP-2 and MMP-9 by Western blotting

Further quantification was obtained by direct comparison of samples from the same patient’s veins on Western blots. The intensity of staining over the area of each band was measured by densitometric scanning and again expressed as a percentage of the HT1080 internal standard. Although less sensitive and more statistically variable, Western blotting confirmed that surgical preparation significantly increased the level of all MMP proteins secreted into conditioned media over the first 48 h (Fig. 2). Likewise, the large-fold increases in the levels of the 68 kDa active MMP-2 and the 95 kDa pro-MMP-9 produced by culturing veins in serum for 12 days were confirmed by Western blotting, with no difference between the freshly isolated and surgically prepared veins. The smaller-fold changes in pro-MMP-2 levels after culturing were not detectable by Western blotting.

### 3.4. In situ hybridization

MMP-2 mRNA was detected in luminal (Fig. 3A,B) and adventitial microvessel (data not shown) endothelial cells and some medial SMC of both freshly isolated and surgically prepared vein segments before culture (Fig. 3A,B) or after 48 h serum-free culture (data not shown). After culture in serum for 12 days, MMP-2 mRNA expression was present at similar intensities in neointimal cells

![Image](https://academic.oup.com/cardiovascres/article-abstract/33/2/447/379171)
and microvessels endothelial cells and medial SMC in freshly isolated and surgically prepared vein segments before culture (Fig. 4A,B) or after 48 h serum-free culture (data not shown). After culture in serum for 12 days, MMP-9 mRNA was prominent in both freshly isolated and surgically prepared veins, especially in the neointimal SMC (Fig. 4C). MMP-9 mRNA expression was also detected at high levels in the SMC and microvasculature endothelial cells of the deep media of cultured freshly isolated and surgically prepared vein segments.

Pre-hybridization with a 10-fold excess of unlabelled riboprobe (results not shown) and hybridization of the sections with the $^{35}$S-labelled sense RNA probes for MMP-2 and MMP-9 (Fig. 3D,E and Fig. 4D,E) yielded the expected negative results, demonstrating the specificity of these RNA probes.

### 3.5. Immunocytochemistry for MMP-2 and MMP-9 protein

MMP-2 protein was detected in all cells and also bound to the extracellular matrix of freshly isolated veins before (Fig. 5A) or after 48 h serum-free culture (results not shown). Similarly high levels of MMP-2 protein were detected after surgical preparation (Fig. 5B). After culture in serum for 12 days, equally high levels of MMP-2 protein were detected in all cell types of both vein types (Fig. 5C,D).

A low level of MMP-9 protein expression was detected in freshly isolated vein segments before culture (Fig. 6A) and after 48 h in serum-free media (results not shown). Although expression was predominantly localized in luminal and microvascular endothelial cells, $55 \pm 10\%$ (mean $\pm$ s.e.m., $n = 6$) of medial SMC had detectable staining before culture. Surgical preparation increased the intensity and widened the distribution of MMP-9 protein expression (Fig. 6B) either before or after (results not shown) 48 h serum-free culture. Significantly more medial SMC stained for MMP-9 in surgically prepared veins ($89 \pm 5\%$, $n = 6$, $P < 0.05$ vs. freshly isolated). After culture in serum for 12 days, intense staining for MMP-9 protein was detected in neointimal SMC of both vein types (Fig. 6C,D). Positive medial SMC were also detected in both vein types, but again in significantly higher numbers in surgically prepared ($89 \pm 3\%$, $n = 6$) vs. freshly isolated ($67 \pm 11\%$, $n = 6$) veins.

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Fig. 2. Measurement of MMP-2 and MMP-9 in conditioned media by Western blotting. Conditioned media were collected from organ cultures of freshly isolated (Fi) or surgically prepared (SP) vein over the first 2 days of culture (0–2) and in the 2 days after culture with serum for 12 days (12–14). MMP expression was measured by densitometric scanning of Western blots (O.D. × mm$^2$) and expressed as a percentage of the internal HT1080 standard (mean ± s.e.m., $n = 6$). (a) 68 kDa active gelatinase A. (b) 72 kDa pro-gelatinase A. (c) 95 kDa pro-gelatinase B. * $P < 0.05$ vs. freshly isolated days 0–2 (Student’s $t$-test). No significant difference in gelatinolytic activity was detected between freshly isolated and surgically prepared vein at days 12–14.

Fig. 3. Detection of MMP-2 mRNA expression in vein segments before and after culture using in situ hybridization. (A) One of 6 freshly isolated vein segments before culture. (B) A surgically prepared vein segment from the same patient. (C) The surgically prepared vein segment after 14 days in culture with serum. (D) Serial section to panel A hybridized with $^{35}$S-labelled MMP-2 sense RNA probe. The scale bar in panel A applies to all panels and represents 50 μm. The arrowheads indicate positive endothelial cells; large arrows indicate the intima and media boundary.

Fig. 4. Detection of MMP-9 mRNA expression in vein segments before and after culture using in situ hybridization. (A) One of 6 freshly isolated vein segments before culture. (B) A surgically prepared vein segment from the same patient. (C) The surgically prepared vein segment after 14 days in culture with serum. (D) Serial section to panel A hybridized with $^{35}$S-labelled MMP-9 sense RNA probe. (E) Serial section to panel C hybridized with $^{35}$S-labelled MMP-9 sense RNA probe. The scale bar in panel A applies to all panels and represents 50 μm. The arrowheads indicate positive endothelial cells; large arrows indicate the intima and media boundary.
Fig. 7. Immunocytochemistry-negative controls. Immunocytochemistry of a typical surgically prepared vein segment after 14 days in culture with serum using (A) MMP-2 antisera which has been pre-adsorbed with the MMP-2 peptide and (B) MMP-9 antisera which has been pre-adsorbed with the MMP-9 peptide. The scale bar in panel A applies to both panels and represents 25 μm.

$P < 0.05$ vein segments. The staining for MMP-2 and MMP-9 was abolished by pre-adsorption of the antibody with the corresponding synthetic peptide, demonstrating the specificity of this technique Fig. 7A,B).

4. Discussion

4.1. MMP expression by freshly isolated vein

Bands of lysis in gelatin zymograms at 95, 72 and 68 kDa were positively identified as the proforms of gelatinase B and A and the active form of gelatinase A, respectively, in agreement with studies of rabbit aorta [13]. The rate of SMC proliferation is barely detectable in freshly isolated veins [29] and secretion of MMP-9 was correspondingly low. MMP-9 mRNA and protein expression was mainly confined to luminal and microvascular endothelial cells. By contrast, secretion of pro-MMP-2 was readily observed and MMP-2 mRNA and protein expression was detected in all cell types, confirming previous findings of constitutive expression in isolated SMC [26,39].

4.2. Effect of surgical preparation on MMP expression

Surgical preparation resulted in partial endothelial loss and injury to medial SMC (decreased ATP concentration), in agreement with previous work [6]. This injury results primarily from distension, while adventitial removal and storage for up to 3 h do not affect ATP concentration [40] or prostacyclin production (endothelial function) [41]. Surgical preparation significantly increased secretion of pro-
MMP-9 over the first 48 h. The effect was highly consistent with a coefficient of variation for these stimulated values of approximately 20%. Immunocytochemistry for MMP-9 showed that the increase in MMP-9 expression in medial SMC occurred within 3 h before veins were placed in serum containing medium. We conclude that injury per se induces the expression of MMP-9. Our conclusion is consistent with data showing increased MMP-9 expression after wounding of isolated SMC cultures [42] and after balloon injury [16,18].

Secretion of pro-MMP-2 was also increased significantly by surgical preparation. High-level MMP-2 mRNA and protein expression was located in all cell types. Surgical preparative injury increased production of the active 68 kDa MMP-2, consistent with data on balloon injury [16,18].

4.3. Effect of culturing in serum on MMP expression

Secretion of pro-MMP-9 from freshly isolated and surgically prepared vein segments was significantly increased after culture in serum for 12 days. Significantly more MMP-9 positive medial cells were detected in the surgically prepared than freshly isolated veins. The increased expression of MMP-9 shown here was associated with a higher rate of medial smooth muscle cell proliferation (18 ± 3 vs. 8 ± 4%) after 12 days of culture with serum, consistent with the permissive role proposed for gelatinases in this process [13]. Predominant expression of MMP-9 in neointimal SMC of both vein types again demonstrated an association with SMC migration and proliferation [6].

A significant increase in pro-MMP-2 secretion was also detected with zymography, although not by Western blotting, probably due to its lower sensitivity (200-fold) and greater variability. Significantly increased production of active 68 kDa MMP-2 was detected by both assays. MMP-2 mRNA and protein remained distributed at high levels in all cell types; hence activation of MMP-2 rather than increased expression is likely to be the key factor in facilitating SMC proliferation and migration.

4.4. Further implications

The mechanisms responsible for increased MMP-9 expression following injury and during neointima formation are unknown. Growth factors including PDGF, tumour necrosis factor-α and interleukin-1 (IL-1) are known to upregulate MMP expression in human isolated SMC [26,39], while PDGF and IL-1 synergistically increase MMP-9 expression in rabbit aortic SMC [36]. PDGF mRNA and protein expression are increased by culturing in neointimal SMC in this model [4]. PDGF may therefore be one factor mediating increased MMP-9 protein expression during culture, although this remains to be tested directly. However, surgical preparation does not increase PDGF expression in this model and so other factors must also be involved. The method of proteolytic activation of MMP-2 in human saphenous vein is presently unknown.

In conclusion, these results provide new insight into the mechanisms underlying the effects of surgical preparative injury and culture on human SMC proliferation. They provide strong evidence that increased expression and activation of basement-membrane-degrading MMPs is an important component of the response to surgical preparative injury and culturing in serum. Based on previous inhibitor studies [13,16,17,19], increased expression of MMP-9 and activation of MMP-2 are likely to be involved in facilitating the SMC migration and proliferation that contribute to neointima formation. Inhibition of MMP activity is therefore a promising therapeutic strategy for vein graft intimal thickening.

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References


