Expression of Tissue Inhibitor of Metalloproteinase-1, -2, and -3 During Neointima Formation in Organ Cultures of Human Saphenous Vein

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Abstract—Degradation of the extracellular basement membrane is implicated in atherosclerosis, restenosis after angioplasty, and intimal thickening of vein grafts. Upregulation of metalloproteinase (MMP)-2 and MMP-9 accompanies neointima formation in cholesterol-fed rabbits, in rat and pig models of angioplasty, and in organ cultures of human saphenous veins. MMPs are inhibited by binding to tissue inhibitors of MMPs (TIMPs). Relatively little is known about their regulation in relationship to neointima formation; thus, we investigated TIMP expression in the organ culture model. Qualitative reverse transcriptase–polymerase chain reaction of mRNA extracted from veins showed that TIMP-1, TIMP-2, and TIMP-3 are each expressed before and after culture. Zymography revealed that TIMP-1 was the most abundant TIMP secreted and that its secretion increased dramatically between 0 to 2 and 12 to 14 days of culture. An enzyme-linked immunosorbent assay showed that TIMP-1 secretion increased from 3.2±1.5 (mean±SE) to 32±6 ng/mg wet weight per day (n=5, P<0.01). Immunocytochemical testing localized the increased expression of TIMP-1 to neointimal smooth muscle cells. Although less abundant, TIMP-2 secretion also increased from 0.8±0.3 to 4.7±0.2 ng/mg wet weight per day (n=5, P<0.001), and tissue levels increased from 33±7 to 150±70 ng/mg wet weight (P<0.05). TIMP-2 was also immunolocalized to neointimal smooth muscle cells and their surrounding matrix. TIMP-3 was not secreted but was detected variably and constitutively in tissue extracts (160±120 and 170±100 ng/mg wet weight [n=9] on days 2 and 14, respectively). TIMP-3 was found in the cells and extracellular matrix of the media and adventitia before culture and to a lesser extent in the neointima after 14 days of culture. Rates of total TIMP secretion on day 14 exceeded those of MMP-2 and MMP-9 (10.6±1.9 and 15.6±2.3 ng/mg wet weight per day, respectively). Consistent with this, in situ zymography showed that MMP gelatinase activity was highly localized to cell bodies in the media and neointima. Secretion of TIMP-1 and TIMP-2 is greatly increased during neointima formation in human saphenous veins. TIMP-1 is readily released, whereas TIMP-2 remains partially attached and TIMP-3 exclusively attached to the extracellular matrix. Regulation of TIMP expression is therefore an important determinant of net MMP activity during neointima formation, restricting it to the pericellular environment. (Arterioscler Thromb Vasc Biol. 1999;19:255-265.)

Key Words: vascular smooth muscle cells ■ coronary artery bypass grafting ■ atherosclerosis ■ metalloproteinases ■ proliferation

Aortocoronary bypass grafting with autologous veins is a common procedure to improve blood flow in patients with severe coronary stenosis. However, its long-term success is limited by occlusion in up to 50% of grafts after 10 years. Occlusion results from neointimal thickening, favoring later superimposition of atherosclerotic plaques. Histological and experimental studies have shown that migration and proliferation of vascular smooth muscle cells (SMCs) play key roles in neointima formation in vein grafts. These events are also implicated in the pathogenesis of atherosclerosis and restenosis after balloon angioplasty. Recent work demonstrates that turnover of the extracellular basement membrane is a critical regulator of SMC migration and proliferation. In support of this concept, basement membrane–degrading metalloproteinases (MMP-2 and MMP-9) are produced by vascular cells (including SMCs, especially when dedifferentiated), by macrophages, and by endothelial cells (ECs). Secretion of MMP-9 from SMCs is stimulated by growth factors and inflammatory cytokines implicated in pathological vascular conditions. Pharmacological inhibition of MMP activity decreases both migration and proliferation of cultured rabbit SMCs. Increased MMP-2 and MMP-9 activity occurs in the aorta of cholesterol-fed rabbits and in rat and pig models of carotid angioplasty. Inhibitor studies demonstrate direct involvement of MMPs in SMC migration and proliferation in vivo. In the context of vein graft stenosis, increased expression of MMP-9 and activation of MMP-2 have been observed during neointima formation in organ cultures of human saphenous veins and in a pig model of vein grafting. Inhibitor studies of organ cultures confirm that MMPs are functionally involved in neointima formation.

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**TIMP Expression in Human Saphenous Vein**

In addition to transcriptional regulation and activation of pro-forms, MMP activity is strictly controlled by binding to endogenous tissue inhibitors of MMPs (TIMPs). The possibility exists, therefore, that MMP and TIMP levels are both regulated during neointima formation and influence the balance of extracellular matrix (ECM) synthesis and degradation. However, relatively little has been published about TIMP expression in pathological vascular conditions. Importantly, no previous study has systematically compared TIMP and MMP activities during neointima formation. The TIMPs that have been characterized in vascular tissue (TIMP-1, TIMP-2, and TIMP-3) are equipotent in inhibiting active MMPs but differ in their ability to bind the proenzymes at a distinct C-terminal site.\(^{19}\) TIMP-1 (28 kDa) binds to and inhibits activation of pro-MMP-9, whereas TIMP-2 (21 kDa) either promotes\(^ {20}\) or inhibits\(^ {21}\) activation of MMP-2, depending on its concentration. Transcriptional regulation is one mechanism controlling levels of TIMPs in the ECM. TIMP-1 is secreted as a soluble protein; its activity is strongly inducible by inflammatory cytokines in some cell types, including fibroblasts, although it is constitutively expressed by cultured human and rabbit SMCs.\(^ {6,9}\) Immunohistochemical studies in human atherosclerotic plaques have produced conflicting results. Nikkari et al\(^ {22}\) found increased expression in plaques, whereas Galis et al\(^ {23}\) found similar levels in atherosclerotic and normal tissue. Studies conducted after balloon injury have also produced conflicting results. In rabbit aortas, relative increases in TIMP-1 messenger RNA and protein levels in the neointima were demonstrated,\(^ {24}\) whereas in rat carotid arteries, an increase in TIMP-2 but not TIMP-1 was demonstrated.\(^ {25}\) In none of those studies\(^ {22–25}\) were TIMP-1 levels quantified absolutely or compared with MMP levels. The importance of TIMP-1 expression in neointima formation is therefore unclear. The significance of TIMP-2, which is also soluble and expressed constitutively in isolated SMCs,\(^ {6,9}\) is similarly unclear. Galis et al\(^ {23}\) found equivalent levels of immuno-reactive TIMP-2 in atherosclerotic and normal tissues. However, Hasenstab et al\(^ {26}\) showed a relative increase in functional TIMP-2 levels 3 to 7 days after balloon injury to rat carotid arteries. TIMP-3 (22 kDa) is not secreted freely but remains tightly bound to the ECM;\(^ {26}\) its activity in rabbit SMCs is induced by platelet-derived growth factor (PDGF) and transforming growth factor-\(\beta.\)\(^ {7}\) Its role in neointima formation has not been investigated.

This study was designed to define quantitatively the expression of TIMP-1, TIMP-2, and TIMP-3 during neointima formation in an organ culture of human saphenous vein and to compare it with expression of basement membrane–degrading MMP-2 and MMP-9. This model was chosen because of the well-characterized kinetics of neointima formation\(^ {27}\) and expression of MMP-2 and MMP-9,\(^ {16}\) the availability of antibodies to each of the 3 human TIMPs, and the relevance of the model to coronary artery bypass grafting.

### Methods

**Vein Collection and Culture**

Human saphenous vein segments were obtained from 23 patients (mean age \(\pm SD\), 54 \(\pm 4\) years) undergoing coronary bypass surgery. Ethical approval was obtained from the relevant authority. Veins were cultured by a modification of the method of Pederson and Bowyer\(^ {28}\) as described previously.\(^ {27,29}\) In brief, immediately after careful dissection from the patient’s leg, the segments were placed in warm (30°C to 37°C) RPMI 1640 medium containing 20 mmol/L HEPES buffer, 0.225 mg/L papavine hydrochloride, 5 mg/mL amphotericin B, and 20 IU/mL sodium heparin for transport to the tissue culture laboratory. Within 10 to 30 minutes, the vein segments were placed in warm (20 mmol/L HEPES-buffered RPMI 1640 containing 2 mmol/L L-glutamine, 8 mg/mL gentamicin, 100 IU/mL penicillin, and 100 mg/mL streptomycin), and excess fat and adventitia were removed. The vein was then cut longitudinally and pinned with the endothelial surface up in segments of 5 to 10 mm in length. Veins were cultured at 37°C in bicarbonate-buffered RPMI 1640 supplemented with 30% FBS unless otherwise stated under an atmosphere of 95% air and 5% CO\(_2\).

**RT-PCR**

For initial qualitative assessment of TIMP mRNA expression, RT-PCR was performed on paired vein segments before and after 14 days of culture. Vein segments were ground under L\(_2\)N\(_2\), and total RNA was isolated by centrifugation through a CsCl gradient after the tissue and cells were lysed with 4 mol/L guanidinium isothiocyanate.\(^ {30}\) One microgram of RNA was reverse-transcribed in a reaction mixture containing 1× reverse transcriptase buffer (50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl\(_2\), 10 mmol/L DTT, 2 \(\mu\)L RNasin, 1 mmol/L dNTPs, 12.5 mg/mL random hexamer primers, and 200 units of Moloney murine leukemia virus reverse transcriptase. Samples were incubated for 60 minutes at 42°C and at 95°C for 4 minutes. Finally, 80 \(\mu\)L of water treated with diethyl pyrocarbonate was added to create the cDNA pool. Ten nanograms of the cDNA pool was amplified by PCR using primers specific to TIMP-1, TIMP-2, and TIMP-3, and GAPDH as follows: \(\text{RT-PCR:}\) sens, ACCCAACAGCGGGCTTCTCGAATTC; anti-sense, GGCTATCTGGGACCGCAGGGACTGC; TIMP-2: sens, TGGAATGACATGATGATGATCAGGG; anti-sense, TGCTTATGGCTCCTGATGTCGAA; TIMP-3: sens, CTACACCATCACGAGATGGAATG; anti-sense, TGGGGCATTGATTGTGCCTTTATCC; and GAPDH: sens, ACCCTTCTAGATGACTCATCACTACA; anti-sense, ATGACAGCAGATCACCTGCTGACCAT-CAT. Each reaction was subjected to 40 cycles (denaturing at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute). The identity of PCR products was assessed by ICN Biomedicals: FBS, from Advanced Protein Products; papavine hydrochloride, from McCarthy Medical; and gentamicin, from Roussel. Reverse transcriptase–polymerase chain reaction (RT-PCR) reagents were obtained from Perkin Elmer Cetus. The ELISA kit for TIMP-1, enhanced chemiluminescence Western blotting detection reagents, and LM-1 photographic emulsion were purchased from Amersham International. Polyvinylidene difluoride membranes were purchased from Bio-Rad. HT1080 fibrosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD). Recombinant human MMP-2 and MMP-9 were obtained from TCS Biologicals (Buckingham, UK). Recombinant human TIMP-1, TIMP-2, and TIMP-3 and antibodies to TIMP-1 and TIMP-2\(^ {28,29}\) were provided by Dr Gillian Murphy (Strangeways Laboratories, Cambridge, UK). The TIMP-2 but not the TIMP-1 antibody recognizes the corresponding bovine TIMP. Both antibodies immunoprecipitate complexes containing MMPs, indicating that they recognize MMP-TIMP complexes as well as unbound TIMPs.\(^ {27}\) Antibodies to TIMP-3 were provided by Dr K. Iwata (Fuji Chemicals Ltd, Takaoka, Japan) and are described elsewhere.\(^ {30}\) The antibody recognizes TIMP-3 from several species, including humans and cattle. The rabbit anti-sheep biotin-conjugated antibody was obtained from The Binding Site (Birmingham, UK), and the biotin-conjugated rabbit anti-mouse, rabbit anti-sheep, and rabbit anti-mouse peroxidase-conjugated antibodies were supplied by Dako (High Wycombe, Buckinghamshire, UK). Vectashield mounting liquid was purchased from Vector Laboratories.

**Materials**

All reagents except those listed below were obtained from Sigma Chemical Company. The MMP inhibitor, Ro 31-9790, was a gift from Roche Products Ltd. Sodium heparin was obtained from CP Pharmaceuticals; L-glutamine, penicillin, and streptomycin, from Gibco BRL; RPMI 1640 culture media and amphotericin B, from Amersham International. Penicillin, streptomycin, and L-glutamine were obtained from TCS Biologicals (Buckingham, UK). Recombinant human TIMP-2 and MMP-9 were obtained from TCS Biologicals (Buckingham, UK). Recombinant human TIMP-1, TIMP-2, and TIMP-3 and antibodies to TIMP-1 and TIMP-2\(^ {28,29}\) were provided by Dr Gillian Murphy (Strangeways Laboratories, Cambridge, UK). The TIMP-2 but not the TIMP-1 antibody recognizes the corresponding bovine TIMP. Both antibodies immunoprecipitate complexes containing MMPs, indicating that they recognize MMP-TIMP complexes as well as unbound TIMPs.\(^ {27}\) Antibodies to TIMP-3 were provided by Dr K. Iwata (Fuji Chemicals Ltd, Takaoka, Japan) and are described elsewhere.\(^ {30}\) The antibody recognizes TIMP-3 from several species, including humans and cattle. The rabbit anti-sheep biotin-conjugated antibody was obtained from The Binding Site (Birmingham, UK), and the biotin-conjugated rabbit anti-mouse, rabbit anti-sheep, and rabbit anti-mouse peroxidase-conjugated antibodies were supplied by Dako (High Wycombe, Buckinghamshire, UK). Vectashield mounting liquid was purchased from Vector Laboratories.
size fractionation of ethidium bromide–stained agarose gels and by sequence-specific hybridization to 32P–γ-labeled internal oligonucleotide probes after Southern blotting of gels (GAPDH internal probe, GTCCTCCACCATCGGAG; TIMP-1 probe, TCTTGCACAT-CACTACTGTG; TIMP-2 probe, CTATTGCCAGGAAGGGCCA; and TIMP-3 probe, CTGTGCACATCTTGAGGAG). 

Zymography and Reverse Zymography

MMP-2 and MMP-9 secretion was quantified by zymography as described previously13,16 using authentic human proteins (0.4 ng) as standards. For analysis of functional TIMP secretion, paired segments of the same veins were cultured for either 0 to 2 days in serum-free medium or 12 days in serum containing culture medium followed by 2 days in serum-free medium after extensive washing. Vein segments were then weighed. The conditioned media were removed, concentrated 40-fold, and stored at −20°C before analysis. Reverse zymography was performed essentially as described by Ward et al.13 Eight microliters of each sample (each pair of samples was adjusted for equal wet weight by diluting the more concentrated sample with distilled water) was electrophoresed at 4°C through an 11% (wt/vol) polyacrylamide gel containing 0.2% (wt/vol) SDS supplemented with 1 mg/mL gelatin. After removal of SDS, the gels were incubated for 1 hour at 37°C in conditioned medium from PMA-activated rabbit skin fibroblasts (which contain a mixture of activated MMPs), followed by 16 hours in incubation buffer (50 mmol/L Tris, pH 8.0, 50 mmol/L NaCl, 10 mmol/L CaCl2, and 0.05% of Brij 35). Inhibition of MMP activity by TIMPs leads to darker bands against the lighter background after Coomassie blue staining. Recombinant human TIMPs (40 ng per lane) were used as standards.

Quantification of TIMP Production

TIMP-1 secretion was quantified using a sandwich-based ELISA according to the manufacturer’s instructions; the assay appears to be highly specific for human TIMP-1 but cross-reacts with TIMP-1–MMP complexes. The assay produces a linear curve from 0 to 50 ng/mL of human TIMP-1. Conditioned media samples were diluted to fall within this linear range. Samples were assayed in duplicate. TIMP-2 and TIMP-3 were quantified by Western blot analysis. For TIMP-2, 20 μL of 40× concentrated media (each pair adjusted for equal wet weight as described above) was electrophoresed on an 11% (wt/vol) polyacrylamide gel containing 0.2% (wt/vol) SDS supplemented with 1 mg/mL gelatin. After removal of SDS, the gels were incubated for 1 hour at 37°C in conditioned medium from PMA-activated rabbit skin fibroblasts (which contain a mixture of activated MMPs), followed by 16 hours in incubation buffer (50 mmol/L Tris, pH 8.0, 50 mmol/L NaCl, 10 mmol/L CaCl2, and 0.05% of Brij 35). Inhibition of MMP activity by TIMPs leads to darker bands against the lighter background after Coomassie blue staining. Recombinant human TIMPs (40 ng per lane) were used as standards.

In Situ Zymography

In situ zymography was performed essentially as described by Galis et al.23 Unfixed 8-μm frozen sections were cut and air-dried on glass slides at room temperature (23°C). In the dark, LM-1 microautoradiography emulsion (Amersham), which contains 2.3% (wt/vol) gelatin, was diluted with an equal volume of 50 mmol/L Tris-HCl, pH 7.6, 50 mmol/L NaCl, 10 mmol/L CaCl2, and 0.05% Brij 35. The slides containing sections were then coated with a layer of emulsion, placed horizontally in humidified chambers, and incubated for 18 hours at 37°C. After incubation, the emulsion was allowed to air-dry at room temperature. The slides were then developed with Kodak D-19 developer and fixed with Kodak Unifix for 8 minutes. Regions with gelatinase activity appeared as light areas against the black background of the developed emulsion. As controls for the specificity of the gelatinase activity, inhibitors of MMPs (100 μmol/L Ro 31-9790 or 20 mmol/L EDTA) were incorporated into the mixture of buffer and emulsion.

Statistical Analysis

When normally distributed, data were expressed as the mean±SEM and were compared by using a 2-tailed paired Student’s t test. Otherwise, the Wilcoxon signed rank test for pairs was used. Differences were considered significant at P<0.05.

Results

Expression of TIMP mRNA in Saphenous Vein

To obtain an initial indication of the range of TIMPs expressed by human saphenous vein, we undertook qualitative RT-PCR analysis of total RNA extracted from veins. This revealed that TIMP-1, TIMP-2, and TIMP-3 messenger RNAs were all detected in samples both before and after 14 days in culture (Figure 1). RT-PCR for GAPDH confirmed the integrity for all but 1 RNA sample (lane 10); this sample also failed to show TIMP-1 expression. These data established that there was some constitutive expression of each
TIMP in human saphenous veins, but the analysis was not designed to establish whether expression was increased by culturing.

Quantification of TIMP Protein Secretion Into Conditioned Media

To quantify TIMP secretion and the influence of culturing on it, we measured concentrations of TIMP proteins in conditioned media. These were obtained from paired vein segments from the same patients after either 0 to 2 days in serum-free culture or after 12 days in 30% serum followed by 2 days in serum-free culture. This protocol was adopted to avoid interference of TIMPs derived from bovine serum or other proteins in subsequent assays of human TIMPs. With the use of reverse zymography, the inhibitory activities of MMP at 29 kDa (equivalent to the electrophoretic mobility of the TIMP-1 standard) were visible after both 0 to 2 and 12 to 14 days in culture (Figure 2). TIMP-1 levels were clearly elevated in the conditioned media from days 12 to 14 compared with the paired sample collected from days 0 to 2 in culture. Bands at 22 kDa (equivalent to the electrophoretic mobility of the TIMP-2 standard) were barely detectable or absent when measured by this technique. To ensure that the elevation in functional TIMP-1 levels was not due to residual contamination from FBS, the same procedure was performed on samples of conditioned media from veins rendered nonviable by pretreatment at 56°C for 20 minutes before culture. We previously showed and confirmed here that this treatment reduced cell viability as measured by ATP concentration from 280 ± 20 to 39 ± 17 nmol/g wet weight. No TIMP-1 was secreted at days 0 to 2 or 12 to 14 from veins pretreated with heat (data not shown).

TIMP-1 secretion was quantified by ELISA, which revealed an elevation from 3.2 ± 1.5 (SE) to 32 ± 6 ng/mg wet weight per day when days 0 to 2 and 12 to 14 in culture were compared (n=5, P<0.01). The TIMP-1 ELISA is specific for the human protein, confirming that the increased TIMP-1 expression was not due to carryover from FBS.

Notwithstanding the inadequate sensitivity of reverse zymography to measure TIMP-2 production, TIMP-2 secretion was quantified by Western blotting (Figure 3A) of concentrated conditioned medium from veins cultured for 2 or 14 days. Two-day cultures were conducted in serum-free medium throughout, whereas 14-day samples were from veins cultured for 12 days in 30% serum followed by 2 days in serum-free medium. Loading of pairs of samples was normalized according to the wet weight.

Reverse zymography of conditioned media

Figure 2. Reversed gelatin zymogram. The first lane shows 40 ng of standard human TIMP-1 (conditioned medium from rabbit SMCs infected with an adenovirus that drives human TIMP-1 and standardized by ELISA) (T1, 28 kDa); the second lane, 100 ng of recombinant human TIMP-2 (T2, 21 kDa); and subsequent lanes, conditioned medium (concentrated 40×) from 5 vein pairs cultured for 2 or 14 days. Two-day cultures were conducted in serum-free medium throughout, whereas 14-day samples were from veins cultured for 12 days in 30% serum followed by 2 days in serum-free medium. Loading of pairs of samples was normalized according to the wet weight.
TIMP-2 secretion was observed from 0.8±0.3 to 4.7±0.2 ng/mg wet weight per day when days 0 to 2 and 12 to 14 in culture were compared (n=5, P<0.001). TIMP-2 levels in media from veins rendered nonviable by pretreatment at 56°C for 20 minutes before culture were not detected by Western blotting (data not shown), demonstrating that the increase was due to endogenously produced TIMP-2. TIMP-3 was not detected by Western blot analysis in the conditioned medium after either 0 to 2 or 12 to 14 days (data not shown). This result was expected because TIMP-3 associates with the ECM.

Quantification of TIMP Levels in Tissue Extracts
TIMP-2 is known to associate with cell membranes through binding to membrane-type MMPs.7,8 Thus, 1 reason for the lower levels of TIMP-2 secretion might be sequestration within the vein tissue. We therefore conducted Western blot analysis on vein tissue extracts. TIMP-2 was detected in total protein extracts (Figure 3B), and densitometric quantification showed an increase from 33±7 to 150±70 ng/mg wet weight when days 0 to 2 and 12 to 14 were compared (P<0.05, Wilcoxon test). This is equivalent to a linear rate of accumulation within the tissue of (150−33)/12=10 ng/mg wet weight per day between 2 and 14 days. TIMP-2 levels in extracts of heat-pretreated veins cultured for 14 days were 6.4±2 ng/mg wet weight (n=3), confirming that the increase was due to endogenously produced TIMP-2. TIMP-3 was also detected in total protein extracts from vein segments, but this was highly variable from patient to patient (Figure 4; 3 data sets not shown). In 5 pairs of samples, TIMP-3 levels appeared to increase after culture; in 4 others, it appeared to decline. The mean levels were 160±120 and 170±100 ng/mg wet weight (n=9) after 2 and 14 days in culture, respectively, which was not significantly different when the data were compared by using a paired Wilcoxon test. TIMP-3 levels in extracts of cultured, heat-pretreated veins were 1.8±0.9 and 0.2±0.12 ng/mg wet weight, respectively.

Location of TIMP-1, TIMP-2, and TIMP-3
Immunocytochemical testing revealed distinct patterns of expression of individual TIMPs during development of the neointima in the organ culture model. Given the evidence detailed above that TIMP-1 is readily secreted from the tissue, we used pretreatment with monensin to trap the protein within the endosomal compartment of cells.29 TIMP-1 was not detected by immunoperoxidase staining in medial cells in vein samples cultured for 0 to 2 days (Figure 5A), whereas TIMP-1 was located in many of the neointimal cells but few medial SMCs after 14 days in culture (Figure 5C). At high-power magnification (Figure 6A), punctate staining for TIMP-1 could be seen within individual cells, demonstrating endosomal trapping. Identification of the TIMP-expressing cells as SMCs and surface ECs was confirmed by immunocytochemical testing using α-SMC actin and QBend-10, respectively (data not shown). Punctate intracellular staining for TIMP-1 was not detected in the adventitia either before or after culturing (Figure 5B and 5D) or in cells at the cut surfaces (not shown).

In sections from the same blocks, light, diffuse staining for TIMP-2 was located extracellularly in the medial and adventitial layers of veins culture for 0 to 2 days (Figure 7A and 7B). Much stronger cell-associated staining was observed after 14 days (Figure 7C and 7D). The greatest intensity of staining for TIMP-2 was in the neointima; however, increased staining was also seen in medial and adventitial cells (Figure 7C and 7D). Increased staining of the ECM was also apparent.

TIMP-3 was detected extracellularly in the upper region of the media, apparently sparing the endothelial layer in samples at day 2 (Figure 8A and 8B). There was also diffuse staining of the ECM in the media and adventitia (Figure 8B). After 14 days, TIMP-3 was just detectable in the ECM of the neointima but remained prominent in the upper media (Figure 8D). The staining was strongest below the internal elastic lamina at the border between the neointima and media. Scattered staining of the medial and adventitial ECM remained. Overall, staining intensity varied considerably in samples of veins from different patients, consonant with the Western blotting data.

Comparison of TIMP and MMP Levels in Cultured Veins
We previously documented a 6-fold increase in MMP-2 secretion and a 20-fold increase in MMP-9 secretion from human saphenous veins during culture.16 However, absolute values of gelatinase secretion were not recorded. To achieve this, we used zymography standardized with authentic human recombinant proteins. In the same veins used for estimation of TIMP secretion, MMP-2 and MMP-9 secretion between 12 and 14 days of culture was 11±2 and 16±2 ng/mg wet weight per day (n=5), respectively. Similarly to TIMP-1, most MMPs were found in the medium. After 14 days of culture, the tissue concentrations of MMP-2 and MMP-9 were 1.8±0.9 and 0.12±0.06 ng/mg wet weight, respectively.

Location of Gelatinase Activity
In situ zymography was used to evaluate gelatinase activity in veins cultured for 2 days and showed prominent activity at the luminal surface of ECs (large arrows in Figure 9B) and in the deep media. There was also lower-level activity in the inner media and adventitia. In veins cultured for 14 days, activity remained highest in the deep media (Figure 9D). However, activity in the inner media was greatly increased (Figure 9D) and appeared to extend beyond individual cells. Activity in the neointima and adventitia was comparatively less and closely associated with cell bodies (large arrows in Figure 9D) rather than the intervening ECM. These patterns of activity were greatly suppressed by EDTA and the specific MMP inhibitor (Ro 31-9790; data not shown).

Figure 4. Western blot analysis of TIMP-3. A, Tissue extracts from 2 paired segments of veins cultured for 2 or 14 days was analyzed for TIMP-2. B, Tissue extracts from a second series of 4 paired vein segments.
We demonstrated qualitatively that human saphenous vein expresses messenger RNAs for TIMP-1, TIMP-2, and TIMP-3. Secretion of TIMP-1 and TIMP-2 into conditioned media was increased 10-fold and 6-fold, respectively, by culturing in serum. After culturing, TIMP-1 was 7-fold more abundant in conditioned medium than TIMP-2, and TIMP-3 was not secreted. TIMP-2 and TIMP-3, but not TIMP-1, remained bound to the tissues. Tissue levels of TIMP-2 were increased almost 5-fold by culturing, whereas levels of TIMP-3 did not change. Total levels of both secreted and matrix-associated TIMPs exceeded the corresponding levels of gelatinases. Immunocytochemical testing showed that TIMP-1 was secreted selectively by neointimal SMCs. TIMP-2 was increased after culturing not only in neointimal SMCs but also in cells of the media and adventitia. TIMP-3 remained bound to the tissues. Tissue levels of TIMP-2 were increased almost 5-fold by culturing, whereas levels of TIMP-3 did not change. Total levels of both secreted and matrix-associated TIMPs exceeded the corresponding levels of gelatinases. Immunocytochemical testing showed that TIMP-1 was secreted selectively by neointimal SMCs. TIMP-2 was increased after culturing not only in neointimal SMCs but also in cells of the media and adventitia. TIMP-3

**Figure 5.** Immunoperoxidase staining for TIMP-1 on frozen sections. Paired monensin-treated veins were obtained from the same patient after 2 and 14 days in culture. A, Only background staining could be detected in either surface cells or the media after 2 days. B, Adjacent field showing background staining of the adventitia. C, TIMP-1 was detected throughout the whole neointima after 14 days in culture. D, Adjacent section showing less cellular TIMP-1 staining in the adventitia. A indicates adventitia; IE, internal elastic lamina; M, media; and NI, neointima (determined from serial sections stained with Van Gieson’s stain). The scale bar represents 25 μm.
was present in the neointima after culturing but was more prominent in the matrix of the media and adventitia. In situ zymography demonstrated that gelatinase activity was greatest in the media; it was highly restricted in the neointima, implying a functional role of neointimal expression of TIMPs.

The data provided here are the first quantitative estimates of TIMP expression in human vascular tissue. Our observation of basal secretion of TIMP-1 and TIMP-2 by normal veins is consistent with histological evidence from nonatherosclerotic arteries. The quantitative demonstration of upregulation of TIMP-1 is an important finding. TIMP-1 secretion was unequivocally localized to human neointimal SMCs by demonstrating its entrapment by monensin within endosomal vesicles. Increased expression of TIMP-1 by neointimal cells is consistent with results of the histological studies of Nikkari et al in carotid artery tissues. However, other histological studies of human atherosclerotic arteries failed to show upregulation of TIMP-1 or TIMP-2, perhaps because of the greater sensitivity of their antibodies in detecting basal levels. Our data showing upregulation of TIMP-1 in the neointima are also consistent with results of recent studies showing relative increases in the balloon-injured rabbit aorta.

Previous experiments, including our own, of TIMP-1 secretion using isolated SMCs have demonstrated constitutive expression at the mRNA and protein levels. Isolated cultured cells probably reflect the properties of synthetic-phenotype SMCs, similar to those found in the rapidly developing neointima. A large number of genes are known to be upregulated in neointimal SMCs, and TIMP-1 appears to fall into this category, as shown in this study. It is possible that activation of the TIMP-1 gene occurs during phenotypic modulation of the contractile medial cells into synthetic-state neointimal cells, perhaps by occupation of activator protein (AP)-1 and polyomavirus enhancer-A binding protein-3 transcription elements. This hypothesis is worthy of future investigation. Recent evidence indicates that myofibroblasts derived from the adventitia may constitute a proportion of the cells of the neointima of vein grafts in pigs and in organ cultures of human saphenous vein. However, under the culture conditions we use, we have not observed migration of cells from the cut edges of tissue pieces. Moreover, when

Figure 6. Immunoperoxidase staining for TIMP-1. A, Nonimmune IgG control corresponding to the sections in Figure 5C after 14 days in culture. The scale bar represents 25 μm. B, Higher magnification (100×) reveals intracellular granules of TIMP-1 protein (large arrows) in neointimal cells. The scale bar represents 10 μm. IE indicates internal elastic lamina; M, media; and NI, neointima (determined from serial sections stained with Van Gieson’s stain).
ECs and inner medial SMCs were selectively labeled before culture by infection with an adenovirus that drives expression of β-galactosidase, many of the neointimal cells that developed after culture were found to express β-galactosidase. This supports the conclusion that at least some cells arose by migration and division of the underlying SMCs. In the current experiments, only neointimal cells were observed to secrete amounts of TIMP-1 that were detectable by immunocytochemical testing. TIMP-1 secretion was not detected in either resident adventitial cells or in cells at the cut edges of cultures. Hence, our data do not support the involvement of adventitial fibroblasts. However, the general lack of definitive cell-specific markers does not allow us to rule out some contribution.

We showed quantitatively, also for the first time, that secretion of TIMP-2 is upregulated during organ culture of human saphenous vein. Immunocytochemical testing for TIMP-2 revealed increased staining in neointimal cells and media after 14 days in culture, compared to the early stages. This indicates a role for TIMP-2 in neointima formation, possibly due to its role in regulating angiogenesis and extracellular matrix remodeling.
human vascular tissue, despite a constitutive pattern of secretion from isolated SMCs. Our data are consistent with the relative changes in TIMP-2 expression in the rat carotid artery that occur after balloon injury. Regulation of TIMP-2 expression is less well understood than that of TIMP-1. The promoter of the human TIMP-2 gene also contains both AP-1 and polyomavirus enhancer-A binding protein-3 sites, although the AP-1 site may be inactive. Unlike TIMP-1, a significant proportion of TIMP-2, equivalent to a linear rate of production of 10 ng/mg per day, accumulated in the matrix, compared with secretion of ≈5 ng/mg per day. Part of this may be bound to membrane-type MMPs, which are present in human saphenous veins (A. Kranzhofer and A.C. Newby, unpublished data, 1998). Overall, the rate of accumulation of TIMP-2 (10 + 5 = 15 ng/mg wet weight per day) was approximately half that of TIMP-1 (32 ng/mg wet weight per day). The finding of accumulation of TIMP-2 in the neointima is consistent with data from studies of rat carotid arteries.

Figure 8. Immunocytochemical testing for TIMP-3. Frozen sections of veins were obtained after 2 and 14 days in culture. A, TIMP-3 is found extracellularly in the subendothelial layer and throughout the media. B, Staining extends to the adventitia. C, After 14 days in culture, TIMP-3 is just detectable between the cells of the neointima and remains prominent in the media, especially near the internal elastic lamina. D, There is also clear staining of the adventitia. A indicates adventitia; IE, internal elastic lamina; M, media; and NI, neointima (determined from serial sections stained with Van Gieson’s stain). The scale bar represents 25 μm.
Figure 9. In situ zymography. The zymograms show the location of active gelatinases in human saphenous vein. A, Section from a vein cultured without serum for 2 days; the section was coated with photographic emulsion and incubated for 18 hours at 37°C. Areas of gelatinase activity appear as clear spaces against the black background. Note the focal areas of high activity associated with the luminal ECs and in the deeper media and areas of less activity in the inner media and adventitia; IE, internal elastic lamina; M, media; and NI, neointima. B, Higher magnification shows focal areas of high activity associated with the luminal ECs (large arrows). C, Section from a vein incubated in serum for 14 days; the section was coated with photographic emulsion and incubated for 18 hours at 37°C. Note the abundance of activity in the media extending to the inner media and areas of less activity in the adventitia and neointima. D, Higher magnification shows restriction of activity to cell bodies (large arrows) within the neointima. A indicates adventitia; IE, internal elastic lamina; M, media; and NI, neointima (determined from serial sections stained with Van Gieson’s stain). The scale bar in A and C represents 50 μm. The scale bar in B and D represents 25 μm.

In rabbit aortic SMCs, TIMP-3 expression was stimulated by PDGF and/or transforming growth factor-β. PDGF expression is detectable in human saphenous veins even before culture, which may explain the basal expression of TIMP-3. Consistent with this, TIMP-3 was prominent in the subendothelial matrix, and ECs are the most abundant source of PDGF in veins before culture.47

Our study is also the first to compare directly the levels of TIMPs and gelatinases during neointima formation. The clear conclusion is that rates of gelatinase and TIMP secretion are of the same order. Moreover, the large relative increases in gelatinase expression and activation previously reported are matched by similar increases in expression of TIMP-1 and TIMP-2. Hence, regulation of TIMP expression must have a major impact on net MMP activity, especially in the neointima. By immunoprecipitation, both the TIMP-1 and TIMP-2 antibodies recognize the unbound TIMP and TIMP-MMP complexes.29 The same data cannot be obtained for the TIMP-3 antibody because TIMP-3 is insoluble. However, the epitope recognized by the antibody is the C-terminal tail of TIMP-3, which is not thought to be involved in binding to MMPs.48 It is likely that this antibody also recognizes both unbound and complexed TIMPs. Hence, in situ zymography is the only available method to investigate the balance between TIMPs and MMPs. This demonstrated that in the neointima, where immunohistochemically detected MMP and TIMP levels are high (George et al46 and these studies), gelatinase activity was nevertheless closely restricted to the pericellular environment. Experiments with synthetic MMP inhibitors and gene transfer of TIMPs support an essential role of active MMPs in permitting migration of SMCs.4,15,49 –51 Two of these studies also found an inhibitory effect on early proliferative responses to serum or injury.3,15 If unopposed, however, the increased secretion of gelatinases would lead to uncontrolled neointimal expansion and prevent the establishment of a stable ECM between the neointimal cells. Mechanisms are therefore necessary to limit the activity of gelatinase and other MMPs that degrade interstitial matrix components, such as collagenase and stromelysin. The up-regulation of TIMP-1 and TIMP-2 that occurs in neointimal SMCs appears to satisfy this requirement for a self-protecting mechanism. In other pathological conditions in which MMPs are involved, such as wound healing, there is strong evidence of concomitant or consequent upregulation of TIMPs that modulates the increased MMP activity.52 Our results clearly indicate that the same principle applies to neointima formation in blood vessels.

In conclusion, our data demonstrate important changes in the expression and location of TIMP-1, TIMP-2, and TIMP-3 during neointima formation in human saphenous veins. The abundance of TIMPs relative to gelatinases demonstrates that these changes will have an important impact on activity and hence influence turnover of the neointimal ECM. The net effects will likely be to restrain SMC migration and proliferation and to promote establishment of a stable ECM.

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Expression of Tissue Inhibitor of Metalloproteinase-1, -2, and -3 During Neointima Formation in Organ Cultures of Human Saphenous Vein
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