Differential expression and activity of matrix metalloproteinases during flow-modulated vein graft remodeling

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Objective: **While shear stress closely regulates vascular remodeling, the mediators of this process have been only partially elucidated. The current study examined the role of the gelatinases in flow-mediated vein graft intimal hyperplasia. We hypothesized that matrix metalloproteinase (MMP)–2 and MMP-9 expression and protein levels, relative to tissue inhibitors of metalloproteinase (TIMP)–1 and TIMP-2, are upregulated in a flow-dependent manner during vein graft arterialization.**

Methods: **Bilateral common carotid interposition vein grafting was performed in rabbits. Reduction in flow was achieved through unilateral ligation of the internal carotid artery and three of four branches of the external carotid artery. At 28 days grafts were harvested and analyzed for intimal area; MMP-2 and MMP-9, and TIMP-1 and TIMP-2 messenger RNA content, via quantitative reverse transcription polymerase chain reaction; and MMP-2 and MMP-9, and TIMP-1 and TIMP-2 protein concentrations, via both bioactivity assay and zymography.**

Results: **Branch ligation resulted in a 10-fold difference in mean flow rate and accelerated development of intimal hyperplasia in a low-flow environment. Exposure of the vein graft to arterial hemodynamics induced a marked rise in MMP-9 mRNA levels, whereas only a modest increase in MMP-2 mRNA was observed. MMP-2 protein was 50 to 100 times more abundant than MMP-9, and was significantly upregulated in grafts that demonstrated enhanced intimal thickening. Immunohistochemistry demonstrated that MMP-2 was located throughout the myointima, whereas MMP-9 was localized almost exclusively to the region of endothelium. No differences in TIMP-1 and TIMP-2 mRNA or protein levels were detected between high-flow and low-flow grafts.**

Conclusion: **MMP-2 is the predominate gelatinase that regulates early vein graft remodeling. Despite a marked increase in MMP-9 gene expression, development of intimal hyperplasia after a reduction in wall shear rate correlates with an increase in MMP-2 protein levels. These data suggest differential regulatory mechanisms for proteases within the remodeling vein graft wall. Modulation of extracellular matrix biologic features may offer therapeutic strategies for the prevention of vein graft failure. (J Vasc Surg 2004;39:1084-90.)**

Development of vein graft intimal hyperplasia is initiated by the migration of smooth muscle cells (SMCs) into the intima, and is sustained by the proliferation of these cells in this subendothelial region.[1,2] Breaching of the internal elastic lamina and deposition of matrix in the developing neointima are integral to this process.[3] This balance between matrix synthesis and degradation, as mediated by local protease activity, controls the rate and magnitude of hyperplasia development.

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While many factors have been proposed to regulate the architecture of vein grafts, biomechanical forces are integral to the process.[4,5] Vein graft adaptation under arterial forces proceeds via a combination of intimal thickening and expansive remodeling, in which local alterations in wall shear regulate the balance between these two processes and determine the morphologic characteristics of the graft. Yet to be fully elucidated are the mediators through which intimal thickening predominates under reduced shear conditions; yet expansive remodeling occurs in response to elevations in shear.

Central to the degradation and reorganization of the matrix are matrix metalloproteinases (MMPs), which regulate the process.[6] The relative balance between activated MMPs and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), controls the reorganization of the vein graft in response to exogenous stimuli.[7] Gelatinase A and B (MMP-2 and MMP-9, respectively), as a result of their specificity for elastin and collagen, have been suggested as important regulators of vein graft architecture.[8] The current study examined the differential regulation of the gelatinases and TIMPs in response to shear forces. We hypothesized that MMP-2 and MMP-9 expression and protein levels, relative to TIMP-1 and TIMP-2, are upregu-

RT-PCR primer and probe sequences

	Forward primer	*Reverse primer*	*6FAM-labeled probe*
MMP-2	5′-AGAAGATCGACGCTGTG TACGA-3′	5′-GGCTGAATACACCCAGTA TTCATTG-3′	5′-AGGAGGAGAAGGCCGTG TTCTTTGCAG-3′
MMP-9	5′-CCGGCATTCAGGGAGATG-3′	5′-TCGGCGTTTCCAAAGTACG-3′	5′-TCGGCGTTTCCAAAGTACG-3′
TIMP-1	5′-CGCAGCGAGGAGTTTCTCA-3′	5′-CAAGTCGTGATGTGCAAGAG-3′	5′-CGCTGGACAACTGCGGAACGG-3′
TIMP-2	5′-GAGATCAAGCAGATCAAGA TGTTCA-3′	5′-GGACGGCGCTGTGTAGATG-3′	5′-AGGCCCCGACAAGGACATCGAGT-3′

MMP, Matrix metalloproteinase; *TIMP,* tissue inhibitor of of metalloproteinase.

lated in a flow-dependent manner during vein graft arterialization.

METHODS

Animal model. Eleven New Zealand White male rabbits (3.0-3.5 kg) underwent bilateral carotid interposition vein grafting, with unilateral distal branch ligation, to create defined regions of differential wall shear. Premedication with ketamine (30 mg/kg intramuscularly) was administered, endotracheal tubes were placed, and the rabbits were anesthetized with isoflurane. Heparin (1000 units) was administered intravenously, and the bilateral external jugular veins and common carotid artery were exposed through a vertical midline cervical incision. A 4-cm length of each external jugular vein was harvested for creation of the ipsilateral vein graft. Proximal and distal anastomoses were created end-to-end with a cuff anastomotic technique. For this procedure a rigid polyurethane cuff, fashioned from a 4F vascular sheath (Terumo Medical Corp, Elkton, Md) was used. The end of each jugular vein was passed through a cuff, everted, and fixed with an 8-0 silk ligature. A 2-cm longitudinal arteriotomy was created in the carotid artery, and the cuffed ends of the vein were inserted in a reversed configuration. A second 8-0 silk ligature was used to fix the artery wall around each cuff, and the intervening back wall segment of the carotid artery was excised. Unilateral reduction in graft flow was accomplished through placement of surgical clips, to completely occlude the internal carotid artery and three of the four primary branches of the external carotid artery. Mean blood flow rate through the vein grafts was recorded with an ultrasonic flow meter (2.0-mm probe, T106; Transonic Systems, Ithaca, NY), both before and after ligation of the distal arterial branches.

After 4 weeks the animals were again anesthetized, endotracheal tubes were placed, and heparin was administered. Vein grafts were exposed through a midline neck incision, and bilateral flow measurements were obtained. Segments within 5 mm of the proximal and distal cuffs were discarded, and harvested grafts were fixed in 10% buffered formalin or frozen in liquid nitrogen for protein extraction or messenger RNA (mRNA) isolation.

This study was performed after securing appropriate institutional approval, and conforms with the Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23, revised 1996).

Morphometry and wall shear analysis. Formalin-fixed graft segments (n = 22) were embedded in paraffin, and histologic cross-sections were stained with Masson trichrome and van Gieson elastic stains. Digitized images were collected and analyzed to determine external elastic lamina (EEL) length and neointimal cross-sectional areas for each specimen. EEL diameters were calculated assuming circular in vivo geometry:

$$\text{Diameter} = \text{length}/\pi.$$

. Shear stress was calculated with the Hagen-Poiseuille equation, $\tau = 32 \cdot \mu \cdot Q/\pi \cdot d^3$, where τ is mean wall shear stress, μ is the viscosity of blood (0.035 poise), Q is mean flow rate, and d is the lumen diameter.

Real-time polymerase chain reaction. Quantitative real-time polymerase chain reaction (RT-PCR) was performed on paired vein graft segments (n = 6). Total RNA was isolated with TRI reagent and 1-bromo-3-chloropropane phase separation according to the manufacturer's recommended protocol (Molecular Research Center, Cincinnati, Ohio). After treatment with deoxyribonuclease I (Ambion, Austin, Tex), reverse transcription was completed with random hexamers (PE Applied Biosystems, Foster City, Calif) to obtain a final complementary DNA concentration of 20 ng/μL. TaqMan RT-PCR for the genes of interest (Table) was performed with a 7700 Sequence Detection System (PE Applied Biosystems), using 200 nmol/L of forward primer, 200 nmol/L of reverse primer, 50 nmol/L of probe, and 20 ng of cDNA per 25-μL reaction volume, and TaqMan Universal PCR Master Mix (PE Applied Biosystems). 18S RNA levels were simultaneously determined on all samples for use as an internal control. Both experimental and control samples were assayed in triplicate. The Comparative Ct Method (ΔΔCt Method; User Bulletin No. 2, PE Applied Biosystems) was used for data analysis. Results are expressed as the ratio of target gene mRNA copies to 18S ribosomal RNA copies ($\times 10^6$) to minimize variability from sample loading.

Quantification of total MMP. Paired segments of vein graft (n = 12) were extracted in 0.05 mol/L of Tris (pH 7.5) and 0.2% Triton X-100, with a mortar and pestle. Commercially available MMP-2 and MMP-9 activity assay kits (Biotrack; Amersham Life Sciences, Piscataway, NJ) were used to determine total (combined latent and endogenously active) enzyme concentrations, per the manufacturer's protocol. Before analysis, assay samples were treated

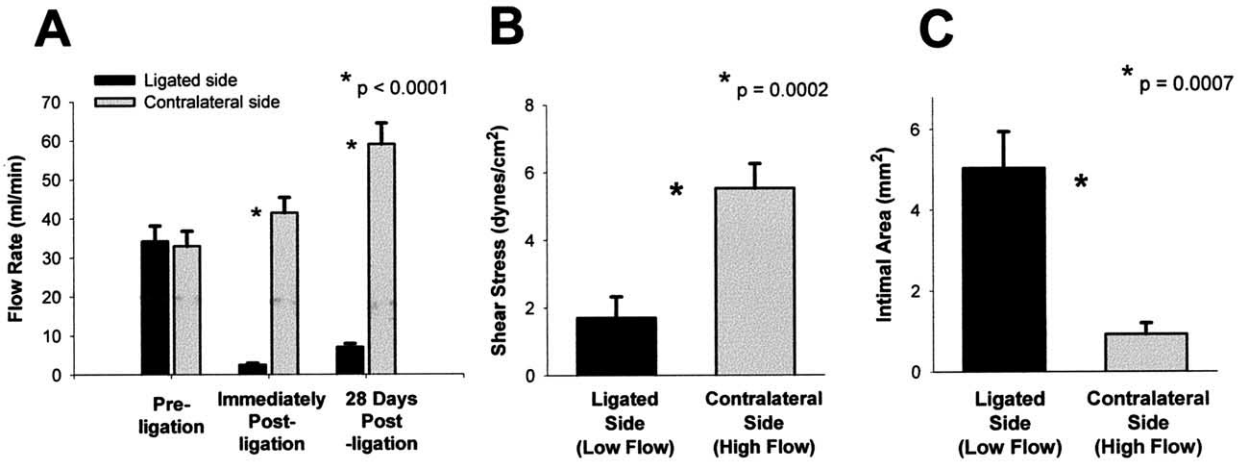

Fig 1. Effect of distal branch ligation on vein graft flow rate **(A)**, shear stress **(B)**, and intimal area **(C)**, 28 days post-ligation.

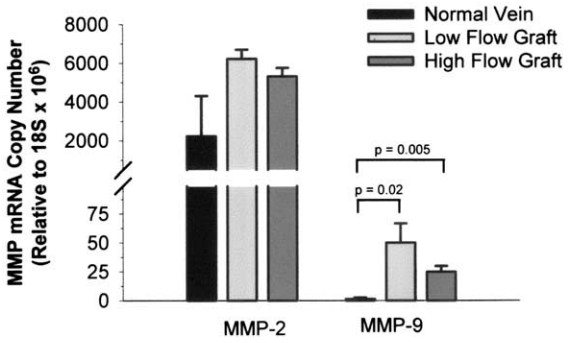

Fig 2. MMP-2 and-9 mRNA expression (left to right) for normal vein, low-flow grafts, and high-flow grafts.

with 0.5 mmol/L (for MMP-2) or 1 mmol/L (for MMP-9) of *p*-aminophenylmercuric acetate; 50-100 μL sample) to activate latent enzyme. MMP-2 and MMP-9 sensitivity ranges were improved to 0.094 ng/mL and 0.31 ng/mL, respectively, by extending the primary incubation time to 20 hours, as described in the manufacturer's technical bulletin. Results are reported in nanograms of total MMP (MMP-2 or MMP-9) per microgram of extracted protein.

Zymography. Protein extraction from paired graft specimens (n = 12) was performed as above. Ten micrograms of protein was separated with 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis containing 0.1% gelatin (Novex; Invitrogen, Carlsbad, Calif). Latent and active forms of MMP-2 and MMP-9 (Oncogene, Cambridge, Mass) were used as positive controls. Gels were renatured and developed with commercially available buffers (Novex). A digital image of the gel was acquired, and band intensity was quantified with one-dimensional Image Analysis Software (Eastman Kodak, Rochester, NY). Band intensity data are presented as the ratio of low-flow to high-flow values.

Quantification of TIMP. Protein extraction was performed as above. TIMP-1 and TIMP-2 commercially available enzyme-linked immunosorbent assay kits were used according to the manufacturer's specifications (Biotrack). Because preliminary data demonstrated that TIMP-1 and TIMP-2 concentrations were below the lower limit of the assay, paired graft samples were pooled (10 μg total protein per sample) from five animals for analysis. Results are reported in nanograms of TIMP (TIMP-1 or TIMP-2) per microgram of extracted protein.

Immunohistochemistry. Formalin-fixed sections were deparaffinized, rehydrated, and treated with MMP-2 (goat anti-human, 1:20; R & D Systems, Minneapolis, Minn) or MMP-9 (goat anti-human, 1:100; R & D Systems) antibodies overnight at 4°C. Assay was completed with the administration of the secondary antibody (rabbit anti-goat), ABC reagent (Vector Laboratories, Burlingame, Calif), and 3,3′-diaminobenzidine (DAB) as a chromogenic substrate. Macrophages were localized with an antibody to RAM-11 (1:200; DAKO, Carpinteria, Calif), with a similar detection scheme. Cell nuclei were counterstained with hematoxylin.

Statistical analysis. All data are expressed as mean ± SEM. Differences in flow rate, shear stress, intimal area, and total MMP-2 and MMP-9 and TIMP-1 and TIMP-2 levels were evaluated with a paired Student *t* test (Sigma Stat, version 2.0). Statistical analysis of band intensity ratios (low-flow to high-flow) obtained at zymography was compared with unity with an unpaired Student *t* test. Differences in gene expression were examined by comparing the cycle number difference, calculated as the difference between the target gene cycle number and 18S cycle number, for low-flow versus high-flow grafts with a paired Student *t* test.

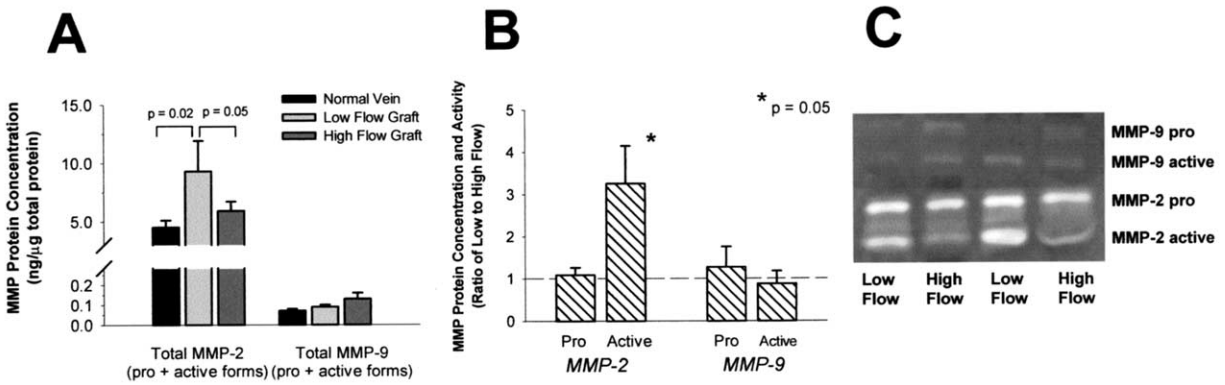

Fig 3. A, Total MMP-2 and MMP-9 concentration (active and latent forms) in normal vein, low-flow grafts, and high-flow grafts. **B,** Relative contributions of latent and active forms, expressed as ratio of low flow to high flow. **C,** Representative zymogram demonstrates low-flow and high-flow grafts.

RESULTS

Hemodynamic parameters and morphologic findings. After placement of bilateral interposition vein grafts, flow rate was approximately equal through both the left-sided and right-sided grafts (Fig 1, *A*). The immediate response to unilateral distal ligation was a marked reduction in flow on the ligated side, with only a minimal compensatory increase in flow on the contralateral side. This initial 10-fold difference in flow rate persisted until graft harvest 28 days later. The effect of these flow changes was a fourfold difference in wall shear stress between the ligated and contralateral grafts (Fig 1, *B*).

Corresponding to the reduction in wall shear was a significant difference in intimal cross-sectional area at graft harvest (5.0 ± 0.9 vs 0.9 ± 0.3 mm^2; $P = .0007$; low-flow vs high-flow grafts, respectively; Fig 1, *C*). No significant difference in the extent of outward remodeling was observed between the two groups (diameter of the external elastic lamina, 4.7 ± 0.4 vs 4.6 ± 0.1 mm; P = NS; low-flow vs high-flow grafts, respectively).

Gelatinase gene expression. Quantitative RT-PCR was used to determine MMP-2 and MMP-9 mRNA expression, with mRNA copy numbers normalized to 18S ribosomal RNA (Fig 2). In freshly harvested segments of the vein MMP-2 mRNA expression was substantially greater than that observed for MMP-9. Notable differences were also observed in vein grafts, where an approximate 150-fold difference between MMP-2 and MMP-9 mRNA levels was detected.

A significant increase in MMP-9 mRNA expression was noted after placement in the arterial circulation for 28 days, with a 40-fold increase in low-flow vein grafts ($P = .02$) and a 20-fold increase in high-flow vein grafts ($P = .005$).

In contrast, a modest twofold to threefold increase in MMP-2 mRNA levels was noted on comparing vein grafts with normal vein (P = NS), and no notable difference in MMP-2 expression for low-flow versus high-flow vein grafts was observed.

Gelatinase protein activity and localization. After addition of *p*-aminophenylmercuric acetate to activate endogenous metalloproteinases, total MMP-2 and MMP-9 levels were determined with a quantitative activity assay (Fig 3, *A*). Corresponding to the observations for gelatinase gene expression, total MMP-2 protein concentrations in normal vein and vein grafts were approximately 100 times greater than total MMP-9.

Vein grafts exposed to low-flow conditions demonstrated a twofold increase in total MMP-2 protein concentration, compared with high-flow vein grafts ($P = .05$) or normal vein ($P = .02$). Total MMP-2 protein level in high-flow grafts was not notably different from that in normal control veins. No significant difference in total MMP-9 protein concentration was observed among low-flow or high-flow grafts or normal control veins.

The relative contributions of latent and active forms of the gelatinases were evaluated with zymography, with data expressed as a ratio of low-flow to high-flow values (Fig 3, *B*). Corresponding to the significant increase in total MMP-2 in low-flow grafts, a threefold increase in the active form of MMP-2 was observed in low-flow versus high-flow samples ($P = .05$). Relative concentrations of pro–MMP-2, pro–MMP-9, and active MMP-9 were not notably influenced by changes in flow.

MMP-2 was identified uniformly within the myointima of the vein grafts, with evidence of enhanced staining within the endothelium (Fig 4). In contrast, MMP-9 was almost exclusively localized to the endothelium. RAM-11 staining revealed only sporadic macrophages within the intima-media (one or two per histologic section), demonstrating that this cell type was not the source of MMP-9 at 28 days. No differences in staining patterns were identified between vein grafts exposed to low or high flow.

TIMP gene expression and protein concentration. Vein grafts demonstrated modest increases in both TIMP-1 and TIMP-2 mRNA expression over normal vein;

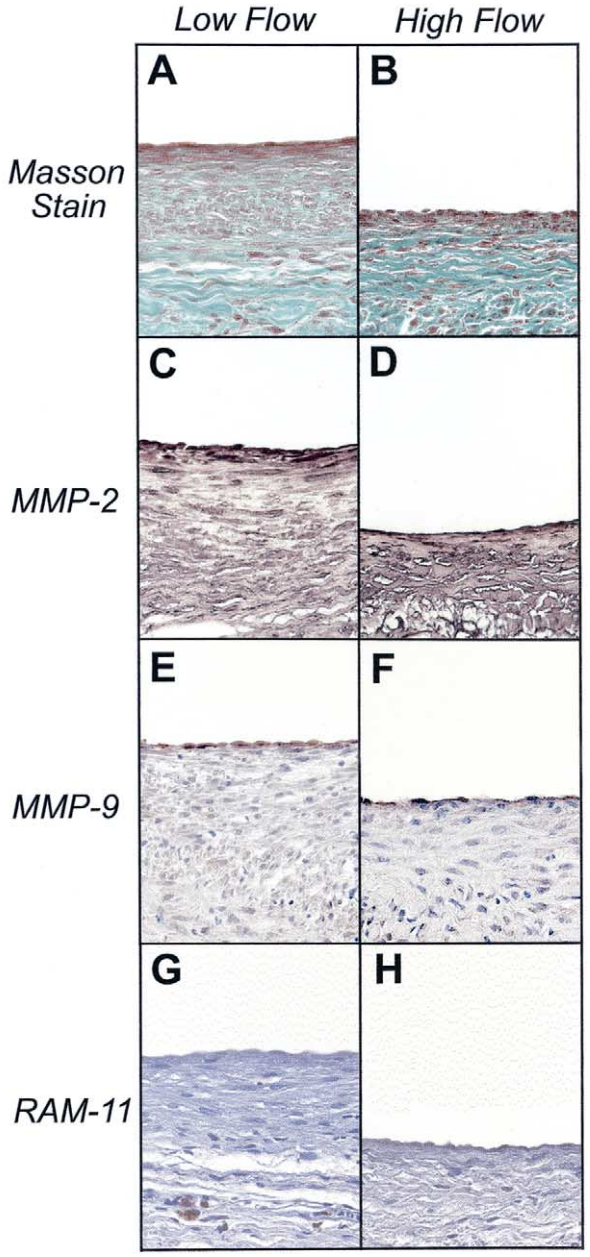

Fig 4. Localization of MMP-2, MMP-9, and RAM-11 in low-flow **(A, C, E, G)** and high-flow **(B, D, F, H)** vein grafts. MMP-2 **(C, D)** is located uniformly in the myointima, with enhanced staining in the endothelium. MMP-9 **(E, F)** is almost exclusively localized to the endothelium. Only sporatic macrophages **(G, H)** are identified, most frequently in the surrounding adventitia. No difference in localization pattern was observed between low-flow and high-flow grafts.

however, these changes did not reach statistical significance (Fig 5, *A*). No differences in TIMP-1 or TIMP-2 mRNA levels (Fig 5, *A*) or protein concentration (Fig 5, *B*) between grafts exposed to high or low flow were observed.

DISCUSSION

We examined the relationship between flow rate, intimal hyperplasia, and gelatinase activity during vein graft remodeling. Consistent with the observations of other investigators, vein grafts exposed to low-flow conditions demonstrated a marked increase in intimal area. Accompanying this change was an increase in MMP-2, predominately in an active form. While an increase in MMP-9 mRNA expression was observed in both low-flow and high-flow grafts, these changes did not translate into significant increases in protein concentration.

SMCs within the wall of the vein are surrounded and embedded by extracellular matrix. On transfer of the vein to the arterial circulation for grafting, the combination of local injury and exposure to altered hemodynamic forces induces an array of signaling mechanisms, which lead to migration and proliferation of the medial SMCs within the developing intima. Essential to the initiation of this process is matrix degradation and remodeling around the migrating SMCs, and an increase in local MMP activity has been suggested as pivotal in this event.[6]

Several investigators have examined the effect of flow on arterial adaptation to alterations in shear. Through use of an arteriovenous fistula, groups have demonstrated that acute elevations in flow lead to an increase in active MMP-2 and MMP-9,[9,10] with pharmacologic MMP inhibition preventing outward remodeling of the artery in response to this increased shear.[9] Potentially conflicting is the observations of Godin et al,[11] in which the mouse carotid artery ligation model was used to examine MMP expression in response to acute reduction in flow. These authors demonstrated that reductions in flow induced a transient increase in MMP-9 expression, peaking at 1 to 3 days, then a delayed increase in MMP-2, starting at day 7 and extending through day 28. Recent data from this group in MMP-9 knockout mice has demonstrated an essential role for MMP-9 in the remodeling process.[12] Lack of the early peak in MMP-9 production resulted in a significant reduction in later thickening of the intima. This temporal pattern of MMP expression in these studies is consistent with our 28-day results in vein grafts, in which we observed an elevation in MMP-2 activity without significant change in MMP-9 activity. While studies in the mouse carotid artery have suggested the importance of a multistep MMP recruitment model,[13] future studies are needed to evaluate the early temporal changes that occur in MMPs, and in particular MMP-9, during vein graft remodeling. Balloon angioplasty, in combination with alteration in flow, has demonstrated similar findings[14]. Angioplasty without changes in flow induced no alteration in MMP-2 expression; however, reductions in flow after balloon injury resulted in a significant increase in MMP-2.

The expression of MMPs has also been examined after transfer of a vein segment to the arterial system for bypass grafting. These studies show that MMP-2 and MMP-9 are increased in vein grafts; however, the temporal expression of this increase has not been clearly defined.[15-18] Expres-

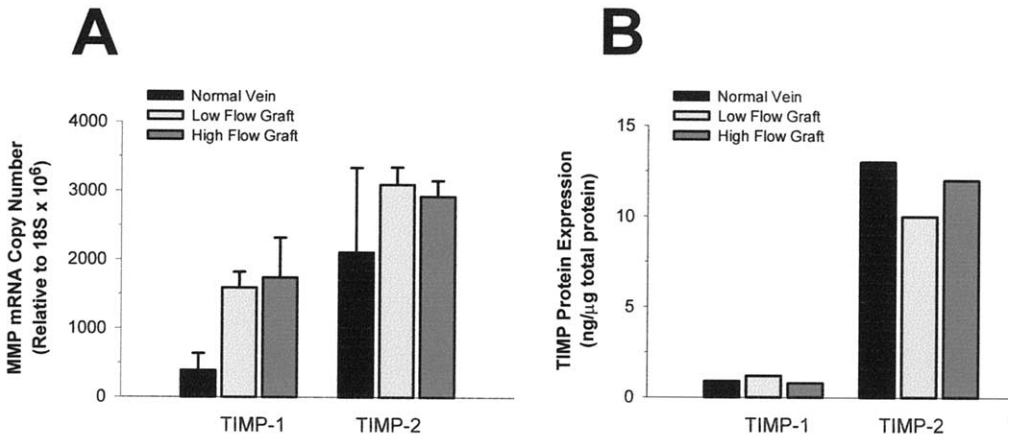

Fig 5. TIMP-1 and TIMP-2 mRNA expression **(A)** and protein concentration **(B)** in normal vein, low-flow grafts, and high-flow grafts.

sion of TIMPs after vein bypass graft placement remains unclear; one study demonstrated an increase in both TIMP-1 and TIMP-2 in vein segments maintained in static culture,[15] whereas no change in TIMP-1 was noted in in vivo vein grafts.[18] The only study to examine the effect of flow on vein graft MMPs was by Patterson et al,[19] in which vein segments were placed in a blood-free perfusion circuit for up to 7 days. Differing from the results we have seen in rabbit vein grafts, these authors observed an elevation in MMP-2 and MMP-9, as well as in TIMP-1 and TIMP-2. Potentially conflicting results may be secondary to differences inherent in the ex vivo perfusion system, for example, nonpulsatile hemodynamic environment, no blood-surface interaction, and potential problems with delivery of nutrition or gas exchange.

In summary, our results are the first to demonstrate that the accelerated intimal thickening that results from a reduction in wall shear is associated with an increase in MMP-2, predominately in an active form. While an increase in MMP-9 mRNA expression was observed in both low-flow and high-flow grafts, these changes did not translate into significant increases in protein concentration. Overall, regulation of gelatinase activity in response to altered wall shear occurs independent of altered TIMP expression.

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