Native Matrix Metalloproteinase Characteristics May Influence Early Stenosis of Venous Versus Arterial Coronary Artery Bypass Grafting Conduits*

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Purpose: Stenosis and occlusion rates of internal mammary artery (IMA) and saphenous vein (SV) coronary artery bypass grafts (CABGs) are markedly different, which result from respective disparities in vascular remodeling. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) regulate vascular structure and may have important influence on graft patency. However, the MMP milieu and expression profile of the IMA and SV have not been contrasted. Therefore, the aim of this study was to assess and compare the native MMP systems in IMA vs SV conduits.

Methods: IMA (n = 10) and SV (n = 10) specimens were obtained from patients undergoing CABG surgery. Protein levels of MMP-1, MMP-2, and MMP-9, TIMP-1, a membrane-bound MMP activator (MT1-MMP), and an extracellular MMP inducer protein (EMMPRIN) were determined by immuno-blotting and quantified by densitometric analysis. MMP-2 and MMP-9 activity was determined by gelatin zymography.

Results: MMP-2 levels were significantly higher in SV (2,218 ± 351 pixels) vs IMA (1,012 ± 213 pixels) specimens (mean ± SEM). There were no significant differences in MMP-1, MMP-9, or TIMP-1 content; however, MT1-MMP and EMMPRIN levels were significantly lower in SV (847 ± 190 pixels, 1,742 ± 461 pixels) vs IMA conduits (2,590 ± 403 pixels, 5,606 ± 678 pixels), respectively (p < 0.05). MMP-9 activity was similar while MMP-2 activity was significantly increased in SV vs IMA specimens.

Conclusions: SV and IMA conduits harbor the same MMP molecular constituents. However, MMP-2 levels and activity are significantly more abundant in the SV compared to the IMA. These differences may contribute to the early pathologic remodeling of the SV vs IMA conduit following CABG surgery.

(CHEST 2004; 125:1853–1858)

Key words: coronary artery bypass grafting surgery; internal mammary artery; matrix metalloproteinase; saphenous vein; vascular conduit

Abbreviations: CABG = coronary artery bypass graft; ECM = extracellular matrix; EMMPRIN = extracellular matrix metalloproteinase inducer protein; IMA = internal mammary artery; MMP = matrix metalloproteinase; MT1-MMP = membrane-bound matrix metalloproteinase; SDS = sodium dodecylsulfate; SV = saphenous vein; TIMP = tissue inhibitor of metalloproteinases; VSMC = vascular smooth-muscle cell

Coronary artery bypass graft (CABG) surgery is the only definitive means for treating coronary artery disease in many patients. The saphenous vein (SV) remains the most commonly utilized conduit for these operative procedures. Unfortunately, the long-term results of CABG surgery are limited by stenosis and subsequent occlusion of SV grafts. Ultimately, the SV generally exhibits unfavorable vascular remodeling properties following arterialization. Intimal hyperplasia is the pathologic hallmark of this pathologic process, resulting in failure rates of 20% and 50% at 5 years and 10 years, respectively. Studies have attempted to elucidate mechanisms underlying intimal hyperplasia within SV conduits. Following CABG surgery, the SV is exposed to increased blood flow and pressure in the arterial system. The resulting alterations in shear and wall stress are thought to contribute to subsequent vasculopathy. Additionally, mechanical stresses associated with vein preparation appear to stimulate intimal hyperplasia.

Intimal hyperplasia requires proliferation and migration of vascular smooth-muscle cells (VSMCs). Prerequisite to VSMC migration is degradation of the extracellular matrix (ECM). This likely...
involves altered expression, production, and regulation of the matrix metalloproteinase (MMP) proteins that break down the basement membrane permitting cell migration.\textsuperscript{13–16} MMP proteins are synthesized as latent proenzymes and activated by serine proteases as well as MMP-2 and membrane-type MMPs.\textsuperscript{16–18} Tissue inhibitors of matrix metalloproteinases (TIMPs) and the MMP/TIMP ratio tightly regulate these activation pathways, thereby influencing ECM production and degradation.\textsuperscript{17,18} Previous work\textsuperscript{6} has demonstrated increased processing, upregulated synthesis, and cellular proliferation of MMP zymogens in vein conduits subjected to arterial conditions. MMP inhibitors, in turn, have been demonstrated to reduce VSMC migration and neointimal hyperplasia in injured arterial segments.\textsuperscript{6,19} MMP subtypes MMP-1, MMP-2, and MMP-9 have been identified as key components in these vascular remodeling processes.\textsuperscript{20} However, the presence and regulation of ECM MMP inducer protein (EMMPRIN) and membrane-bound MMP activator (MT1-MMP) in this paradigm remain to be determined.

The internal mammary artery (IMA) exhibits a striking absence of occlusive lesions, and has far superior patency rates compared to the SV following CAGB surgery. The reason for these unique IMA qualities has not been clearly determined, but is most likely multifactorial. One contributing factor may be related to characteristics of the MMP system. The native MMP milieu may significantly impact the tendency of a vessel for pathologic remodeling. If so, altering the MMP system may serve as a means of attenuating SV remodeling and stenosis. The aim of this clinical investigation was to contrast the native MMP system of IMA and SV conduits in patients undergoing CABG surgery.

\textit{Patient Enrollment and Tissue Collection}

The experimental protocol for this study was approved by the Human Assurance Committee at the Medical College of Georgia. Subsequently, informed consent was obtained from each patient enrolled in this investigation prior to the surgery. IMA (n = 10) and SV (n = 10) specimens were obtained from 10 consecutive patients undergoing CABG surgery. All patients were operated on by the same primary surgeon and assistants. Samples were obtained from the distal portion of the IMA removed to tailor the length of the conduit. Although there was no difference in MMP levels along the length of IMA specimens (data not shown), only the distal portion was used in experiments. SV samples represented the remaining distal segments following each procedure. These arterial and venous specimens were placed in cold Dulbecco modified Eagle medium and kept on ice. Specimens were transferred to the laboratory, where the surrounding fat was carefully removed. The specimens were then rinsed in sterile saline solution, cut into 3-mm segments, and immediately snap frozen. The patients’ preoperative comorbidities and demographics were recorded and entered into a database (Table 1).

\textit{Vascular MMP Extraction}

Frozen artery specimens were homogenized in extraction buffer (1:10, weight/volume) containing 0.15 mol/L NaCl, 20 mM ZnCl\textsubscript{2}, 1.5 mM Na\textsubscript{3}H\textsubscript{2}PO\textsubscript{4}, 10 mM cacodylic acid, and 0.01% Triton X-100. After centrifugation at 4°C for 10 min at speed of 900g, the supernatant was concentrated using a Centricon concentrator (Millipore; Bedford, MA). Samples were centrifuged at 3,000g for 4.5 h at 4°C, and the protein content was measured using Bio-Rad Protein Assay (Bio-Rad Laboratories; Richmond, CA). Samples were stored at −80°C in small aliquots.

\textit{Immunoblotting}

Protein levels of MMP species (MMP-1, MMP-2, MMP-9 and MT1-MMP, and EMMPRIN) were determined by immunoblotting using antibodies specific for each protein. Vascular extracts (20 μg) were diluted to the appropriate loading concentration in sample buffer containing 0.1 mol/L Tris-HCl, 4% sodium dodecylsulfate (SDS) and 0.01% bromphenol blue, and loaded onto

\begin{table}[h]
\centering
\caption{Patient Characteristics*}
\begin{tabular}{lcc}
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Characteristics & Data \\
\hline
Age, yr & 66 ± 13 \\
Male/female gender, No. & 8/2 \\
White/African-American race, No. & 8/2 \\
Diabetes mellitus & 6 (60) \\
Hypertension & 10 (100) \\
Dyslipidemia & 5 (50) \\
Congestive heart failure & 3 (30) \\
Tobacco abuse & 6 (60) \\
Current medications & \\
Nitrates & 6 (60) \\
Beta-blockers & 6 (60) \\
Calcium-channel blockers & 3 (30) \\
Angiotensin-converting enzyme inhibitors & 4 (40) \\
\hline
\end{tabular}
\end{table}

*Data are presented as mean ± SD or No. (%) unless otherwise indicated.
a 10% SDS-polyacrylamide gel. Samples were then separated at 40 mA using a Tris-glycine running buffer (0.2 mol/L Tris-base, 0.2 mol/L glycine, pH 6.8, and 0.1% SDS). The separated samples were transferred to a nitrocellulose membrane in Tris-glycine transfer buffer supplemented with 20% methanol. The immunoblots were blocked for 1 h in blocking-grade powdered goat milk (5%) diluted in 0.2 mol/L Tris-base, 1.4 mol/L NaCl, 0.1% Tween 20, and 0.02% NaN₃. The membranes were then incubated overnight with the primary antibody for each MMP species at dilutions recommended by the manufacturer (Onco-gene Research Products; Cambridge, MA). Bands were visualized using ECL detection kit from Amersham Life Sciences (Arlington Heights, IL). Recombinant MMP proteins were used as positive controls.

**Gelatin Zymography**

Basal MMP activity was detected using gelatin zymography. Vascular extracts (20 μg) were loaded on 10% gelatin zymography gels (BioRad Laboratories) and separated under nonreducing conditions. The gels were then rinsed twice in 2.5% Triton X-100 and incubated overnight (16 h) in substrate buffer containing 50 mM Tris-HCl, 5 mM CaCl₂, and 1 μmol/L ZnCl₂. The activated gels were stained by Coomassie blue R-250 followed by destaining in 55% methanol and 7% acetic acid.

**Data Analysis**

Zymograms and immunoblots were analyzed by densitometric scanning. Lytic bands within the zymograms demonstrated MMP activity at various molecular weights that were analyzed by Gel Pro Image Analysis Program (Media Cybernetics; Silver Spring, MD) and expressed as optical density (pixels). Immunoblot bands corresponding to the known molecular weight of each MMP species were analyzed in a similar manner. The image analysis was performed by an investigator blinded to the identity of the images. Data obtained from densitometric techniques were compared by using analysis of variance. An α level of p < 0.05 was considered to be statistically significant.

**RESULTS**

**MMP Protein Expression**

Patient demographics, comorbidities, and medications are summarized in Table 1. To assess MMP protein levels in CABG conduits, homogenates of IMA and SV samples were analyzed by immunoblotting. Bands corresponding to the molecular weight of MMP-1 (approximately 42 kd), active MMP-2 (approximately 62 kd), and MMP-9 (approximately 82 kd) were detected in all specimens (Fig 1, 2) Both EMMPRIN and MT1-MMP were readily detectable in IMA as well as SV specimens, demonstrating the presence of proteins involved in the induction and activation of MMPs in both conduits.

There were distinct differences between IMA and SV samples. Both MT1-MMP (2,590 ± 403 pixels vs 847 ± 190 pixels, p < 0.05) and EMMPRIN (5,606 ± 678 pixels vs 1,742 ± 461 pixels) expression were significantly elevated in the IMA vs SV specimens, respectively (p < 0.05; Fig 1). MMP-2 levels, however, were significantly higher in SV than in IMA specimens (Fig 1). There were no detectable differences between the expression of MMP-1, MMP-9, and TIMP-1 levels in IMA vs SV conduits (Fig 2).

**Vascular MMP Activity**

To determine the relationship between MMP activity and protein levels, proteolytic activity was
determined by gelatin zymography of IMA and SV samples. MMP activity was detected at molecular weights corresponding to MMP-2 (72 kd and 62 kd) and MMP-9 (92 kd), and a representative gelatin zymogram is shown in Figure 3, left, A. Densitometric analysis of zymographic data (Fig 3, right, B) demonstrated that gelatinolytic activity corresponding to the molecular weight of MMP-2 was significantly higher in the homogenates from SV specimens (89,672 ± 5,643 pixels) than in IMA conduits (47,920 ± 6,112 pixels).

**DISCUSSION**

The SV is the most commonly employed CABG conduit.\(^1,2,4\) Unfortunately, stenosis and occlusion of SV conduits commonly limit long-term results following CABG surgery.\(^2,5\) Progressive intimal hyperplasia characterized by VSMC proliferation and deposition of connective tissue matrix is the culprit for the majority of the occlusive lesions.\(^7,8\) This pathologic process requires ECM breakdown by the MMP system.\(^2,13–15\) Absence of intimal hyperplasia within IMA conduits suggests that their endogenous MMP system harbors features that attenuate this pathologic remodeling. The MMPs are represented by a family of zinc-dependent enzymes, which participate in the constant synthesis and degradation of the ECM.\(^16,17,20–22\) Several species are commonly expressed in the vasculature, including MMP-1, MMP-2, and MMP-9.\(^9,10,20\) Increased ECM protein synthesis, diminished MMP activity, and/or alter-
ations in induction, activation, or inhibitory components may contribute to vascular collagen deposition and intimal hyperplasia. In addition, breakdown of the basement membrane by MMPs allows cell migration leading to the formation of foam cells and atherosclerotic plaques, which contribute to late vein graft failure. Although other clinical investigations have made important observations regarding the MMP system within the vasculature, native MMP systems of IMA and SV conduits have not been directly compared.

This study compared the induction, activation, and inhibitory components of the MMP system in the SV and IMA. The analysis focused on the expression and activity of MMP species that degrade fibrillar (MMP-1) and denatured (MMP-2 and MMP-9) collagen. These subtypes are also expressed abundantly within human vascular tissue.9,10,20–22 We compared IMA and SV specimens from the same group of patients to avoid potentially confounding patient variables that are known to alter MMP activity and related molecular components. Results demonstrated the presence of MMP elements important in vascular remodeling in both the SV and IMA conduits. The lytic activity was higher in SV specimens as compared to IMA specimens, along with higher protein levels of MMP-2. However, the induction (EMMPRIN) and activation (MT1-MMP) components were significantly less abundant in SV than in IMA conduits. The latter findings may have resulted from negative feedback induced by the higher MMP-2 expression and activity within SV grafts. Alternatively, higher expression levels of induction and activation elements native to the IMA may be related to more frequent ECM turnover. The ECM is a dynamic structure that requires constant synthesis and degradation by MMPs.9,10,17 Higher vascular tone and pulse pressures may demand increased ECM turnover. This could explain the increased induction/activation MMP components within the IMA specimens. The comparatively low levels within the SV may signify a more limited requirement for such ECM turnover, which seems plausible in a relatively less dynamic, low-pressure system. One could speculate that these discrepancies contribute to the relative degree of vascular collagen deposition and intimal hyperplasia formation. It remains unclear what degree of down-regulation and/or up-regulation of these MMP elements occurs in these conduits following CAGB surgery.

Earlier work has demonstrated marked increases in MMP activity within SV conduits. Johnson and colleagues11,12 demonstrated dedifferentiation of smooth-muscle cells and increased MMP activity in the SV following surgical manipulation prior to implantation. Vein grafts have also been shown to undergo early vascular remodeling when subjected to arterial conditions.9 The reason higher levels of MMP induction and activation components reside in the IMA vs SV is not entirely clear. The differences should not be related to between-group variables. Such confounding variables were avoided by acquiring both tissue samples from the same patient group. Furthermore, it is unlikely that harvesting techniques explain the discrepancies between IMA and SV specimens. These differences were only detected in the MMP-2 component. The MMP species known to be activated by mechanical stretch (MMP-9) did not differ either in content or activity between IMA and SV specimens.

Laboratory investigations have shown that MMP activation plays an important role in SV remodeling following arterIALIZATION.6 This is associated with an alteration of the VSMC phenotype from a contractile to synthetic type cell.12 The contractile phenotype maintains vascular tone and supports the vessel circumferentially within the outer wall. Remodeling involves migration of the VSMC through the ECM toward the intima, where it is transferred into a synthetic phenotype and participates in formation of ECM leading to intimal hyperplasia.12 The SV has relatively small amounts of VSMCs to provide support. Inhibition of MMP may attenuate migration and transformation of these cells and provide opportunity for VSMC proliferation of the contractile phenotype in the outer wall. In this manner, the SV might acquire characteristics that more closely resemble the IMA while reducing the process of vascular remodeling. Ultimately, the SV would ideally be directed to remodel into a vessel simulating the IMA. Such remodeling would result in a conduit with improved structural support and less tendency for intimal hyperplasia formation. Manipulation of the SV to achieve such an end point is already being explored at the molecular level.23–25

Various molecular approaches aimed at inhibiting intimal hyperplasia have been investigated.21,24,26–28 Selective delivery of transgenes such as TIMP-1 and TIMP-3 into the SV to alter such molecular pathways has been demonstrated feasible in animal studies.29–33 The MMP system may prove an important target for such transgene therapy. The SV could thereby be altered to have a more favorable complement of MMP components that closely mimic the IMA.

In summary, this study demonstrated that both arterial and venous conduits possess a similar MMP framework important for vascular remodeling. Differences in the relative abundance of activation elements are most likely explained by the role of these components under normal physiologic states. These differences in the native MMP milieu may
have important implications on the early impact of surgical manipulation and subsequent exposure to arterial pressures in the SV vs the IMA. However, the exact role of each MMP component in this process requires further investigation. Novel therapeutic approaches may develop that transform the SV conduit into a vessel with IMA qualities and an increased the long-term patency.

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