Injury Induces Dedifferentiation of Smooth Muscle Cells and Increased Matrix-Degrading Metalloproteinase Activity in Human Saphenous Vein

Jason L. Johnson, Guillaume J.J.M. van Eys, Gianni D. Angelini, Sarah J. George

Abstract—Long-term patency of human saphenous vein bypass grafts is low because of intimal thickening and superimposed atherosclerosis. Matrix-degrading metalloproteinases (MMPs) and changes in vascular smooth muscle cell (VSMC) phenotype are thought to be essential for the VSMC migration that contributes to intimal thickening. We examined VSMC phenotype and MMP activity in saphenous veins obtained before and after surgical manipulation. Surgical preparation of the veins significantly increased pro-MMP-1 expression by 2-fold and significantly reduced tissue inhibitor of MMPs (TIMP)-2 expression, whereas MMP-3 and TIMP-1 were unaffected. Furthermore, caseinolytic and gelatinolytic activities measured by in situ zymography were dramatically elevated by injury. The expression of desmin and smoothelin was significantly decreased by injury, whereas vimentin expression was significantly increased. In addition, these changes in phenotype and MMP activity were localized to a subpopulation of VSMCs, the circumferential medial VSMCs. Our data show that surgical preparative injury induces phenotypic modulation of a subpopulation of medial VSMCs to a synthetic phenotype and increases MMP activity. This may favor matrix degradation, VSMC migration, and the subsequent intimal thickening that leads to graft failure. (Arterioscler Thromb Vasc Biol. 2001;21:1146-1151.)

Key Words: smooth muscle cell □ differentiation □ matrix-degrading metalloproteinases □ tissue inhibitors of matrix-degrading metalloproteinases □ smoothelin

A utologous saphenous vein remains the most commonly used conduit for coronary artery bypass grafting, despite long-term patency rates of 50% after 10 years. Late graft failure results from intimal thickening with superimposed atheroma. Intimal thickening occurs because of migration of medial vascular smooth muscle cells (VSMCs) to the intima, where they proliferate and secrete extracellular matrix proteins. Surgical preparation of the saphenous vein injures the blood vessel and contributes to the poor long-term patency (see review by Angelini and Newby).

Medial VSMCs exist in the normal blood vessel wall in the contractile (differentiated) phenotype. These have a “spindle-like” morphology, maintain vessel wall tone, and are rich in contractile and intermediate filament proteins (IFPs). It has been suggested that migrating and proliferating VSMCs have dedifferentiated to a synthetic phenotype, characterized by a reduction in contractile proteins and alterations in IFPs (see review by Owens). Recent studies illustrate that the amounts of the IFPs vimentin and desmin, the cytoskeleton-related protein smoothelin, and the contractile proteins α-smooth muscle (SM) actin, SM myosin heavy chain (SMMHC), and tropomyosin change when VSMCs shift from the contractile to the synthetic phenotype.

Mechanical injury to the blood vessel wall, particularly endothelial damage, is thought to trigger phenotypic modulation of medial VSMCs, shifting them toward the synthetic phenotype (see reviews by Thyberg and colleagues). To enable VSMC migration, remodeling of the basement membrane and of the interstitial collagenous matrix that maintains VSMCs in a quiescent state must occur. Mechanical injury of aortic explants and isolated VSMCs stimulates the production of extracellular matrix-degrading metalloproteinases (MMPs), which are mainly associated with VSMCs of the synthetic phenotype. Injury of rat carotid arteries and human saphenous veins increases the expression of basement membrane–degrading MMP-2 and MMP-9. Furthermore, MMP inhibitors, as well as gene transfer of the endogenous tissue inhibitors of MMPs (TIMPs), have demonstrated the involvement of MMPs in injury-stimulated intimal thickening. Injury also affects TIMP protein levels. TIMP-2 is increased after rat carotid injury in 2 studies. Although these previous studies have demonstrated that dedifferentiation and MMP activity are required for intimal thickening, it is unknown whether these are caused by injury or whether they are colocated.

The present study aimed to examine whether injury of the saphenous vein caused by surgical preparation affects MMP activity and medial VSMC phenotype before its use as a coronary artery vein graft. The expression of the cytoskeletal...
proteins (desmin, vimentin, and smoothelin) and of contractile proteins (α-SM actin, SMMHC, and tropomyosin) was examined to assess VSMC phenotypic modulation. The expressions of MMP-1, MMP-3, TIMP-1, and TIMP-2 and net collagenolytic and gelatinolytic activity were also determined.

Methods

Materials

All reagents were of the highest grade available and were obtained from Sigma Chemical Co, unless stated differently in the text.

Vein Collection

Paired freshly isolated and surgically prepared segments of human saphenous vein were obtained from 13 patients undergoing coronary artery bypass graft surgery. Freshly isolated veins (2 to 3 cm in length) were obtained immediately after excision from the leg, with use of a no-touch technique. After completion of the last proximal anastomosis, surgically prepared veins (up to 10 cm in length) were obtained from the same patients. Surgical preparation involved dissection from the vascular bed, adventitial stripping, side-branch ligation, gentle manual distension at gentle but uncontrolled pressure (up to 600 mm Hg), and storage in heparinized blood at room temperature (23°C) for between 50 and 180 minutes. Surgically prepared veins were obtained 60 to 180 minutes after the freshly isolated veins. Ethical permission was obtained from the United Bristol Healthcare Trust ethics committee (Ref. E2847). The adventitia was removed, and the vein was cut into 5- to 10-mm segments. Segments were snap-frozen, quenched in isopentane, or fixed in 10% (vol/vol) formalin-PBS and embedded in paraffin wax at right angles to the original direction of blood flow.

Immunocytochemistry

Serial 3-μm paraffin sections were dewaxed and rehydrated (n=6 paired segments). Endogenous peroxidase activity was inhibited with hydrogen peroxide. Antigen retrieval was carried out by using either pressure cooking or trypsin digestion. After the sections were blocked with 20% (vol/vol) goat serum in PBS, sections were incubated overnight at 4°C with primary antibodies diluted in 1% (wt/vol) BSA in PBS (mouse anti-myosin 1:75, anti–α-SM actin 1:400, anti-tropomyosin 1:400, anti-vimentin 1:200, anti-desmin 1:50 [DAKO], and anti-smoothelin 1:5; rabbit anti-MMP-1 1:500 and anti-MMP-3 1:1500 [Dr C Long, Pfizer, Sandwich, UK]; and sheep anti–TIMP-1 36 μg/mL and anti-TIMP-2 35 μg/mL [Prof. G. Murphy, University of Norwich, Norwich, UK]). Sections were incubated with appropriate biotinylated secondary antibodies (DAKOL) diluted 1:200 in 1% (wt/vol) BSA in PBS and then with either horseradish peroxidase or alkaline phosphatase–labeled ExtrAvidin (diluted 1:400 in 1% [wt/vol] BSA in PBS). Color was developed with 0.05% (wt/vol) 3,3′-diaminobenzidine or Fast Red TR/Naphthol AS-MX (Fast Red), respectively, and then nuclei were counterstained with Mayer’s hematoxylin. The percentage of positive circumferential and longitudinal cells was counted in four 0.25-mm² fields. A negative control, for which the primary antibody was replaced with mouse, rabbit, or sheep IgG at the same dilution, was always included.

For dual immunocytochemical labeling (n=6 paired segments), vimentin was detected as described above with 3,3′-diaminobenzidine used as substrate. Desmin was then detected as described above except for the addition of avidin- and biotin-blocking solution (Vector Laboratories) in goat serum and primary antibody, respectively. Peroxidase-labeled ExtrAvidin was substituted with alkaline phosphatase–labeled ExtrAvidin, and Fast Red was used as substrate.

Western Blotting

Tissue levels of desmin, vimentin, and smoothelin (n=3 paired segments) and MMP-1, MMP-3, TIMP-1, and TIMP-2 (n=6 paired segments) were quantified by Western blot analysis, as described previously.17 Equal concentrations of proteins were loaded on gels and detected with rabbit anti-human MMP-1 and MMP-3 antisera diluted 1:1000, 50 μg/mL sheep anti-human TIMP-1 and TIMP-2 antibodies, and 2 μg/mL mouse anti-human desmin, vimentin, and smoothelin antibodies. Densitometric scanning was performed by using a Bio-Rad model GS-690 Imaging Densitometer.

In Situ Zymography

Caseinolytic and gelatinolytic activities were located in frozen sections (n=4 paired segments) as described previously.29,30 Controls, for which the incubation buffer was supplemented with 20 mmol/L EDTA, 500 mmol/L of the nonspecific MMP inhibitor UK-231,890 (BB94) and 500 mmol/L of the gelatibase inhibitor UK-181,587 (CT572, both generous gifts from Dr C. Long, Pfizer Limited, Sandwich, UK), 10 μmol/L Ef64, 100 μmol/L pepstatin A, and 1 μmol/L 4-amidinophenylmethanesulfonyl fluoride (APMSF) were included.

Statistical Analysis

Values are expressed as mean±SEM. Mean values of positive cell counts were compared by a 2-way ANOVA and Student-Newman-Keuls post hoc test to detect differences between 2 groups. Mean values from densitometric scanning were compared by using ANOVA and Tukey-Kramer multiple comparisons. Differences were considered statistically significant at P<0.05.

Results

As published previously,31 a high proportion of saphenous veins used for coronary artery bypass grafting have a preexisting thickening. VSMCs in these thickenings are longitudinally orientated, in contrast to the circumferentially orientated medial VSMCs. All saphenous veins used in the present study exhibited such thickening; therefore, VSMCs are referred to as longitudinal or circumferential. Histological assessment of surgically prepared veins revealed patchy disturbance of the vessel architecture, particularly in the circumferential layer (data not shown). It has been suggested that distension of veins before implantation may induce a number of changes,32 including the loss of cell-to-cell and cell-to-matrix contacts, muscle hypertrophy, and proliferation of circumferential VSMCs.33,34

Expression of Cytoskeletal and Contractile Proteins

The contractile proteins SMMHC, α-SM actin, and tropomyosin were detected in 78±1%, 97±2%, and 85±2% of medial VSMCs of freshly isolated veins, respectively. Similarly, SMMHC, α-SM actin, and tropomyosin were detected in 87±2%, 96±1%, and 84±2% of medial VSMCs of surgically prepared veins, respectively. In both vein types, the expression was similar in longitudinal and circumferential VSMCs.

In freshly isolated veins, a small number of longitudinal and circumferential VSMCs, 14% and 20%, respectively, stained vimentin positive (Figure 1A and Table 1). Significantly higher numbers (38%, P<0.01) of vimentin-positive cells were detected in the circumferential VSMCs of surgically prepared veins (Figure 1D and Table 1). In contrast, the percentage of cells expressing smoothelin and desmin was significantly higher in circumferential VSMCs of freshly isolated veins (Figure 1B and 1C and Table 1) than in surgically prepared veins (75% versus 51%, respectively, for smoothelin [P<0.01] and 79% versus 57%, respectively, for desmin [P<0.05]; Figure 1E and 1F and Table 1). The expression of smoothelin and desmin protein in longitudinal
VSMCs was similar in both vein types. No staining was detected in the negative control sections (data not shown).

Dual immunocytochemistry revealed that almost all cells expressed desmin (red) but that less than half of these cells also expressed vimentin (brown, Figure 2A) in freshly isolated veins. Coexpression of these 2 proteins was restricted to circumferential VSMCs. In contrast, in surgically prepared veins, cells expressing only vimentin were observed (Figure 2B and 2C). Furthermore, although lower numbers of desmin-positive cells were present, all desmin-positive cells coexpressed vimentin (Figure 2B). It is also noteworthy that the vimentin-positive/desmin-negative cells in surgically prepared veins were confined to the circumferential VSMCs.

Therefore, our present findings have shown that vimentin is significantly increased only in circumferential VSMCs within the media of surgically prepared saphenous veins. Conversely, these cells had reduced expression of desmin and smoothelin, suggesting that injury to these veins results in activation and deactivation of proteins linked with VSMC dedifferentiation.

Western blotting for desmin, vimentin, and smoothelin proteins confirmed the immunocytochemical results. Despite some variation between samples, significantly less smoothelin (1.34±0.81 versus 4.82±0.73, \( P<0.03 \)) and desmin protein (4.15±0.39 versus 7.11±1.44, \( P<0.05 \)) and significantly more vimentin protein (8.13±0.73 versus 2.73±1.35, \( P<0.03 \)) was detected in surgically prepared veins than freshly isolated veins (Figure 2D and 2E). Variation between samples may be due to differences in the degree of distension and therefore injury, because pressure is not controlled during surgical preparation.

### Localization and Quantification of MMP-1, MMP-3, TIMP-1, and TIMP-2

MMP-1 protein expression was detected in most medial VSMCs in freshly isolated veins (Figure 3A and Table 1) and surgically prepared veins (Figure 3E and Table 1). However, compared with longitudinal VSMCs, significantly greater numbers of circumferential VSMCs (\( P<0.01 \)) were positive for MMP-1 (Table 1). A low level of MMP-3 protein expression was detected in the longitudinal and circumferential VSMCs of freshly isolated (Figure 3B and Table 1) and surgically prepared (Figure 3F and Table 1) veins.

TIMP-1 protein was detected in almost all longitudinal and circumferential cells in freshly isolated (Figure 3C and Table 1) and surgically prepared (Figure 3G and Table 1) veins. TIMP-2 protein was located in almost all longitudinal and circumferential VSMCs of freshly isolated (Figure 3B and Table 1) and surgically prepared (Figure 3H and Table 1) veins. However, significantly less staining was observed in longitudinal (\( P<0.05 \)) and circumferential (\( P<0.001 \)) VSMCs in surgically prepared veins (Figure 3H and Table 1). Interestingly, the number of TIMP-2 positive VSMCs in the circumferential layer was significantly less than that detected in the longitudinal layer (\( P<0.05 \)). No signal was detected when the antibodies were preadsorbed with the respective negative controls (data not shown).

### TABLE 1. Percentage of VSMCs Positive for Vimentin, Smoothelin, Desmin, MMP-1, MMP-3, TIMP-1, and TIMP-2 in Freshly Isolated and Surgically Prepared Veins

<table>
<thead>
<tr>
<th>Positive VSMCs, %</th>
<th>Freshly Isolated</th>
<th>Surgically Prepared</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Longitudinal</td>
<td>Circumferential</td>
</tr>
<tr>
<td>Vimentin</td>
<td>14±3</td>
<td>20±1</td>
</tr>
<tr>
<td>Smoothelin</td>
<td>67±5</td>
<td>75±3</td>
</tr>
<tr>
<td>Desmin</td>
<td>88±2</td>
<td>79±3</td>
</tr>
<tr>
<td>MMP-1</td>
<td>87±3</td>
<td>93±1</td>
</tr>
<tr>
<td>MMP-3</td>
<td>83±2</td>
<td>92±2</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>93±3</td>
<td>96±2</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>99±1</td>
<td>99±1</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=6). Statistical significance was determined by ANOVA and Student-Newman-Keuls post hoc tests.

\(^\star P<0.05\) vs freshly isolated vein longitudinal VSMCs; \(^\dagger P<0.05\) vs freshly isolated vein circumferential VSMCs; and \(^\ddagger P<0.05\) vs surgically prepared vein longitudinal VSMCs.
Western blotting confirmed that surgical preparation significantly reduced TIMP-2 protein expression, whereas TIMP-1 was not statistically affected (Table 2). Interestingly, a significant increase in pro-MMP-1 protein expression was detected in surgically prepared veins compared with freshly isolated veins (Table 2). No difference in active MMP-1 and pro-MMP-3 and active MMP-3 protein expression was detected between the vein types (Table 2).

**Caseinolytic and Gelatinolytic Activity**

In situ zymography revealed areas of gelatin lysis, indicating gelatinolytic activity, throughout the media in all surgically prepared veins, whereas caseinolytic activity was detected only in the circumferential VSMCs (Figure 4A and 4C, n=4). Addition of the MMP inhibitors BB94 (Figure 4E and 4G) and EDTA (data not shown) abolished caseinolytic and gelatinolytic activity. Casein in situ zymography supplemented with a gelatinase-specific inhibitor (CT572) reduced but did not abolish the caseinolytic activity (Figure 4F and 4H) in surgically prepared veins, indicating that MMPs other than gelatinases are responsible for the observed substrate lysis. No caseinolytic activity (Figure 4B) and only low levels of gelatinolytic activity (Figure 4D) were detected in freshly isolated veins (n=4). Addition of other protease inhibitors (E64, pepstatin A, and APMSF) did not affect the observed activity, demonstrating the specificity of this method (data not shown).

**Discussion**

In the present study, we observed that a subset of VSMCs, the circumferential VSMCs, underwent phenotypic modulation after surgical preparation. Furthermore, this coincides with induced MMP activity. This dedifferentiation of VSMCs and MMP activity may contribute to the VSMC migration and proliferation that leads to intimal thickening and late vein graft failure.

Medial VSMC phenotypic modulation, characterized by changes in desmin and vimentin expression, in response to injury is thought to enable migration and proliferation to result in neointimal thickening.35–37 In the present study, we describe decreased desmin and increased vimentin expression, which is associated with the synthetic phenotype,38 in circumferential VSMCs after surgical preparative injury of

**Figure 2.** Effect of surgical preparative injury on vimentin, desmin, and smoothelin protein expression in saphenous vein. A and B, Dual immunocytochemistry for vimentin (brown) and desmin (red) in freshly isolated (A) and surgically prepared (B) veins. C, Immunocytochemistry for vimentin in surgically prepared vein. Small arrowheads indicate desmin-positive cells. Medium arrowheads indicate cells only vimentin positive. Large arrowheads indicate cells positive for desmin and vimentin. Bar in panel C represents 25 μm and applies to all panels (n=6 paired segments). D, Densitometric scanning results of Western blots expressed as optical density (OD) per square millimeter. *P<0.05 compared with freshly isolated vein. Values are mean±SEM (n=3 paired segments). E, Western blot (n=3 paired segments) for smoothelin protein in freshly isolated and surgically prepared veins.

**Figure 3.** Immunocytochemistry for MMP-1, MMP-3, TIMP-1, and TIMP-2 on serial sections from freshly isolated (A through D) and surgically prepared (E through H) veins: MMP-1 (A and E); MMP-3 (B and F), TIMP-1 (C and G), and TIMP-2 (D and H). Brown (A, B, E, and F) and red (C, D, G, and H) colors indicate positive staining. Bar in panel D represents 25 μm and applies to all panels. Lines in all panels indicate the longitudinal and circumferential VSMC boundary. Arrows in panel D indicate representative positive cells, and arrowheads in panel H indicate representative negative cells (n=6 paired segments).
saphenous vein. In addition, we observed a significant reduction in the cytoskeleton-associated protein smoothelin, a unique protein expressed in contractile circumferential VSMCs, in circumferential VSMCs after injury. This further indicates the conversion of VSMCs from the contractile to the synthetic phenotype in this subset of VSMCs after injury. These findings are supported by the detection of less smoothelin expression in vein bypass grafts compared with saphenous vein obtained at postmortem examination.

Focusing on the MMP/TIMP system, we also assessed the expression and proteolytic activity of these enzymes in response to injury, to determine whether an increased ability for matrix degradation coincides with VSMC phenotypic modulation. Using in situ zymography, we demonstrated that this is the case, with increased amounts of gelatinolytic and caseinolytic activity in veins after surgical preparation. The increased collagenolytic and gelatinolytic activity coincided and was colocated with induced phenotypic modulation in circumferential VSMCs. We propose that this increase in MMP activity is, in part, due to the increased expression of MMP-1 and the decreased expression of TIMP-2 observed in circumferential VSMCs. Taken with our previous findings, ie, that MMP-2 and MMP-9 levels are increased after injury, this suggests that the MMP/TIMP balance is shifted to favor proteolytic degradation after injury.

This increase in MMP activity presumably permits matrix remodeling and the increased migration and possibly proliferation that occur in the vein graft after implantation. Although the precise role of the MMPs in inducing VSMC migration is unknown, there are several potential mechanisms. It may remove the physical restraint to permit migration, sever cell-matrix contacts via integrins or cell-cell contacts via cadherins, permit contacts with interstitial matrix components, expose cryptic extracellular matrix sites, or produce extracellular matrix fragments, which stimulate migration or release matrix or cell-bound growth factors.

In summary, we observed that phenotypic modulation of VSMCs and MMP activity induced by injury were located in the outer circumferential layer of the media. It could be postulated that these VSMCs are more susceptible to mechanical stretch injury as a result of vessel distension. This is of note because we have previously demonstrated that intimal thickening induced by VSMC migration and proliferation is significantly greater in surgically prepared veins than in freshly isolated veins and that VSMC proliferation occurs only in the circumferential VSMCs in surgically prepared veins and is absent in freshly isolated veins. This suggests that dedifferentiation of VSMCs and increased MMP activity induced by injury may contribute to the VSMC migration and proliferation that lead to intimal thickening. However, although we have clearly demonstrated that MMP activity is required for VSMC migration in veins, the direct involvement of MMP activity in VSMC proliferation is less clear. We have previously demonstrated that a synthetic MMP inhibitor reduces VSMC proliferation, but the overexpression of TIMP-1, TIMP-2, and TIMP-3 did not affect VSMC proliferation.

Although we have clearly demonstrated that injury increases MMP activity and phenotypic modulation of circumferential VSMCs, we are unable to show directly that they occur in the same cells. This is due to the nature of the methods used. In situ zymography for MMP activity requires coating the section with the substrate; therefore, immunocytochemistry is impossible. However, despite this, we believe that our data provide very compelling evidence that injury induces MMP activity and dedifferentiation of a subset of VSMCs. Furthermore, the present study does not demonstrate that these changes are essential for intimal thickening, but taken together with previous studies, it suggests that injury may initiate some of the processes that occur after implantation and contribute to intimal thickening.

The present study illustrates that injury of the saphenous vein caused by surgical manipulation is sufficient to cause phenotypic modulation of a subset of VSMCs. It highlights that minimization of preparative injury may inhibit intimal thickening by reducing these changes. Furthermore, stabilizing the MMP/TIMP balance with synthetic MMP inhibitors or overexpression

### Table 2. Quantification of MMP-1, MMP-3, TIMP-1, and TIMP-2 in Freshly Isolated and Surgically Prepared Veins by Western Blotting

<table>
<thead>
<tr>
<th>Protein</th>
<th>Freshly Isolated (n=6)</th>
<th>Surgically Prepared (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-MMP-1</td>
<td>4.9±0.8</td>
<td>11.2±2.6*</td>
</tr>
<tr>
<td>Active MMP-1</td>
<td>3.2±1.6</td>
<td>3.5±1.0</td>
</tr>
<tr>
<td>Pro-MMP-3</td>
<td>3.4±1.4</td>
<td>7.6±1.5</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>3.8±1.1</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>2.0±0.7</td>
<td>5.2±1.1</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>4.1±0.6</td>
<td>1.8±0.6*</td>
</tr>
</tbody>
</table>

OD indicates optical density. Values are mean±SEM (n=6 paired segments). Statistical analysis was carried out by ANOVA and Turkey Kramer post hoc tests.

*P<0.05.

Figure 4. Caseinolytic and gelatinolytic in situ zymography on serial sections from surgically prepared (A, B, E, and F) and freshly isolated (C, D, G, and H) veins in the presence of the MMP inhibitors BB94 (E and G) and CT572 (F and H). Caseinolytic activity is observed as black "holes" on a red fluorescent substrate. Gelatinolytic activity is observed as white holes on a black background. White arrows in panel A indicate areas of lysis. Bar in panel H represents 25 μm and applies to all panels (n=4 paired segments).
of recombinant TIMPs may be clinically useful in reducing intimal thickening and late vein graft failure.

Acknowledgments

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References

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