Early Effects of Arterial Hemodynamic Conditions on Human Saphenous Veins Perfused Ex Vivo

Kreton Mavromatis, Tohru Fukai, Matthew Tate, Naomi Chesler, David N. Ku, Zorina S. Galis

Abstract—Exposure to the arterial hemodynamic environment is thought to be a potential trigger for the pathological remodeling of saphenous vein grafts. Using matched pairs of freshly isolated human saphenous vein, we analyzed the early effects of ex vivo hemodynamic conditions mimicking the venous (native) compared with arterial (graft) environment on the key components of vascular remodeling, ie, matrix metalloproteinase (MMP)-9 and MMP-2 and cell proliferation. Interestingly, we found that arterial conditions halved latent MMP-9 (50±11%, $P=0.01$) and MMP-2 (44±6%, $P=0.005$) levels relative to matched vein pairs maintained ex vivo under venous perfusion for up to 3 days. Immunostaining supported decreased MMP levels in the innermost area of arterially perfused veins. Either decreased synthesis or increased posttranslational processing may decrease MMP zymogen levels. Biosynthetic radiolabeling showed that arterial perfusion actually increased MMP-9 and MMP-2 production. When we then examined potential pathways for MMP zymogen processing, we found that arterial conditions did not affect the expression of MT-MMP-1, a cell-associated MMP activator, but that they significantly increased the levels of superoxide, another MMP activator, suggesting redox-dependent MMP processing. Additional experiments indicated that increased superoxide under arterial conditions was due to diminished scavenging by decreased extracellular superoxide dismutase. Arterial perfusion also stimulated cell proliferation (by 220% to 750%) in the majority of vein segments investigated. Our observations support the hypothesis that arterial hemodynamic conditions stimulate early vein graft remodeling. Furthermore, physiological arterial flow may work to prevent pathological remodeling, particularly the formation of intimal hyperplasia, through rapid inactivation of secreted MMPs and, possibly, through preferential stimulation of cell proliferation in the outer layers of the vein wall. **(*Arterioscler Thromb Vasc Biol.* 2000;20:1889-1895.)**

Key Words: matrix metalloproteinase ■ vein graft remodeling ■ hemodynamics ■ redox ■ extracellular superoxide dismutase

Saphenous vein bypass grafting is an important treatment modality for atherosclerotic disease, but it frequently progresses to graft failure, a cause of ischemic morbidity and mortality.[1,2] Development of intimal hyperplasia, through smooth muscle cell (SMC) proliferation and migration,[3,4] diminishes lumen diameter and, in association with thrombosis and atherosclerosis, can lead to total vein graft occlusion.[1,2] Pathological vein graft remodeling also includes medial and adventitial hypertrophy and sclerosis, whose precise consequences remain to be defined.[5] Previous studies[6–10] have shown that SMC proliferation and migration and general tissue reshaping depend on the activity of matrix-degrading enzymes called matrix metalloproteinase (MMP)-2 and MMP-9, known also as gelatinase A and B. These enzymes degrade basement membrane collagens and elastin,[11] presumably freeing cells to move through the extracellular matrix and enabling the remodeling of tissues.

Two main factors traditionally believed to promote vein graft remodeling are surgical preparation injury and exposure to the arterial hemodynamic environment. We and others have shown that vein graft preparative injury increases MMP-9 and MMP-2 levels and vascular cell proliferation[12–14] as well as intimal hyperplasia.[15] The role of MMPs in pathological graft remodeling is also supported by findings that MMP inhibitors reduce vein graft intimal hyperplasia.[16]

Vascular remodeling, which occurs in response to changes in blood flow and pressure, normalizes shear and wall stress.[17] Cyclic mechanical stretch could stimulate the proliferation of venous SMCs, as suggested by in vitro studies.[18] Although pulsatility and increased pressure and flow, experienced by veins in the arterial hemodynamic environment, are thought to contribute to triggering the graft remodeling, their specific effects are somewhat conflicting. Arterial conditions are characterized by increased wall tension, associated with the formation of intimal hyperplasia,[19] as well as by increased flow, thought to decrease intimal hyperplasia.[20,21] In the present study, we pursued the early effects (up to 3 days) of ex vivo hemodynamic conditions mimicking either

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the bypass graft (arterial) or native (venous) environment on gelatinase expression/activation and cell proliferation in matching pairs of human saphenous veins.

Methods

Tissue Preparation

Saphenous vein leftover after coronary artery bypass grafting was obtained after the last proximal anastomosis. To minimize variance, in each perfusion experiment, we compared arterial versus venous perfusion by the use of 2 matching vein segments derived from a single vein specimen (single donor and same preparative surgeon). We selected only undamaged vein specimens, >10 cm in length, 3 to 5 mm in diameter, and with a uniform gross morphology. In some cases, a vein specimen ring was immediately embedded as the nonperfused (fresh) control.

Ex Vivo Saphenous Vein Perfusion

The ex vivo perfusion system has been described in detail elsewhere.[22] Matching vein segments were placed in 2 identical systems, mounted in physiological orientation between 2 cannulas acting as the inflow and outflow perfusion conduits. Total time from operating room to perfusion was <1 hour. The perfusion medium was DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Fisher). Dextran (5%) was added to obtain physiological viscosity (4 cP). Bromodeoxyuridine (BrdU, 10 μmol/L) was added to the culture medium to study cell proliferation. Venous perfusion conditions were as follows: steady flow, flow rate 15 mL/min, and pressure 10 mm Hg. Arterial conditions were as follows: pulsatile flow 90 cycles/min, flow rate 90 mL/min, and pressure 120/80 mm Hg. Static conditions were no flow/no pressure. The perfusion systems were maintained at 37°C in an incubator containing 95% air/5% CO_2. After perfusion, vein ends were discarded, and each segment was divided into equal (5-mm) rings, which were either immediately processed for biochemical or histological assays or cultured.

SDS-PAGE Zymography

Tissues were extracted in ice-cold 10 mmol/L sodium phosphate, pH 7.2, containing 150 mmol/L NaCl (PBS), 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.2% sodium azide. Samples normalized by total protein content (DC Protein Assay, Bio-Rad) were separated by 10% SDS-PAGE containing gelatin (1 mg/mL) in parallel with prestained molecular weight markers (Novex), as previously described.[23] Gelatinases, identified as lytic bands after staining gels with colloidal brilliant blue G, were quantified by use of the Molecular Analyst software program (Bio-Rad).

Western Blotting

Tissue extracts normalized by protein were separated on 10% SDS-PAGE minigels and then transferred onto nitrocellulose (Bio-Rad). MMP-2 and MMP-9 were detected by using commercially available antibodies (Oncogene Science). Extracellular (ec) superoxide dismutase (SOD) was detected by using a polyclonal chicken antiserum (1:1000) generated against the oligopeptide corresponding to amino acids 211 to 228 (GVCGPGLWERQAREHSER), which was generously provided by Dr David Harrison (Emory University), followed by rabbit anti-chicken horseradish peroxidase labeled secondary antibody (1:15000, Jackson Immunoresearch). Cu/Zn SOD was detected with sheep anti-human Cu/Zn SOD antibody (1:1000, Biodesign International), followed by anti-sheep horseradish peroxidase labeled antibody (1:1000, Jackson Immunoresearch). Reaction was developed with the ECL chemiluminescence kit (Amersham), as described previously.[23] Films were quantified by use of the Molecular Analyst software.

MMP-2 and MMP-9 De Novo Synthesis

Pulse metabolic radiolabeling was performed by culturing vein rings in methionine and cysteine-deficient DMEM containing Expre^{35}S^{35}S (50 μCi/10 mL, DuPont-NEN) for 6 hours. Tissue extracts, normalized by total protein, or culture media aliquots, normalized by tissue weight, were incubated with anti-MMP antibodies (1 μg/50 μg) for 2 hours at room temperature. Immune complexes precipitated with protein A–Sepharose (10 mg per sample, Sigma Chemical Co) were separated by SDS-PAGE. Gels were fixed, impregnated with EN^{3}HANCE (DuPont-NEN), dried, and exposed to x-ray film at −70°C. Immunoprecipitated proteins were quantified by use of the Molecular Analyst software program.

Immunohistochemistry

OCT-embedded frozen tissue sections (7 μm) were fixed in acetone. MMP-2 and MMP-9 were detected by 1-hour incubation with 1:200 primary antibodies, followed by a peroxidase-labeled streptavidin-biotin kit (LSAB 2, DAKO) and diaminobenzidine as substrate. Nuclei were counterstained with Gill's hematoxylin (Sigma Chemical Co). Omission of primary antibody served as a negative control. Sections were imaged by use of a Zeiss Axioscope microscope equipped with a computer-based imaging system with ImagePro Plus software (Media Cybernetics).

Proliferating cells were detected with 1:20 anti-BrdU antibody (DAKO), followed by Texas red–conjugated secondary antibody (Jackson Immunoresearch). All nuclei were counterstained with Hoechst. For each vein specimen, 3 sections spaced 200 μm apart were analyzed. In each section, the BrdU-positive (red) nuclei and all nuclei counterstained with Hoechst (blue) were counted in 3 microscopic fields, each encompassing the full thickness of the vessel wall. This method resulted in counting of at least 5000 cells per vein by 2 independent observers. The percentage of BrdU-positive nuclei was calculated and averaged per vein.

In Situ Zymography

Gelatinolytic activity was detected in frozen tissue sections by use of Kodak NTB2 autoradiography emulsion (1:1 in incubation buffer), as described previously.[24]

Superoxide Production Assay

Superoxide production was evaluated in vein rings by lucigenin (5 μmol/L) chemiluminescence,[25] reported to have high specificity, and validated by use of electron spin resonance.[26] Immediately after ex vivo perfusion, rings from matching vein segments were incubated in Krebs-HEPES buffer (mmol/L: NaCl 99.01, KCl 4.69, $CaCl_2$ 1.87, $MgSO_4$ 1.20, K_2HPO_4 1.03, $NaHCO_3$ 25.0, sodium HEPES 20.0, and glucose 11.1) for 30 minutes at 37°C and then transferred to scintillation vials containing lucigenin in Krebs-HEPES buffer. Counts were obtained at 1-minute intervals by use of a scintillation counter (LS 7000, Beckman Instruments, Inc) in out-of-coincidence mode with a single active photomultiplier tube for 20 minutes. Background counts (lucigenin solution alone) were subtracted from the total counts. Some rings were preincubated with Cu/Zn SOD, diphenyleneiodonium, or diethyldithiocarbamate for 30 minutes. For each condition, 2 different rings from each vein segment were measured and averaged.

Localization of Superoxide

Dihydroethidium (Molecular Probes), which in the presence of superoxide is converted to ethidium and intercalates with nuclear DNA, was used to localize and semiquantitatively assess intracellular superoxide in vein tissue sections, essentially as described by Miller et al.[27] Frozen tissue sections (30 μm) were incubated with 2 μmol/L dihydroethidium in Krebs-HEPES buffer at 37° for 30 minutes in the dark. Sections of arterially and venously perfused matching vein segments were incubated in parallel, with images collected with the use of a 585-nm long-pass filter and identical acquisition parameters on a Bio-Rad MRC 1024 argon confocal microscope.

Statistical Analysis

In each experiment, to minimize individual patient variations, parameters measured for veins maintained under arterial conditions

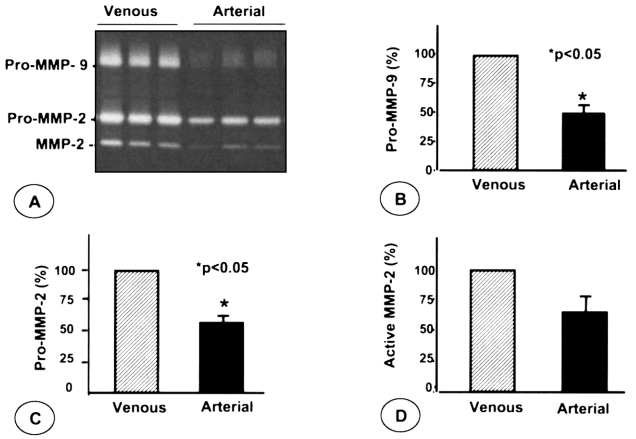

Figure 1. Perfusion under arterial hemodynamic conditions reduces MMP-9 and MMP-2 levels in veins perfused ex vivo for 3 days. A, Representative SDS-PAGE zymography shows MMP-9 and MMP-2 in 3 different samples of matched pairs of venously and arterially perfused veins. B and C, Levels of pro-MMP-9 and pro-MMP-2 (normalized to venous conditions, defined as 100%) show a reduction of both zymogens. D, Levels of active MMP-2 were also reduced in arterially perfused veins compared with matched venously perfused veins. Data are mean±SEM (n=4 pairs of matched vein segments).

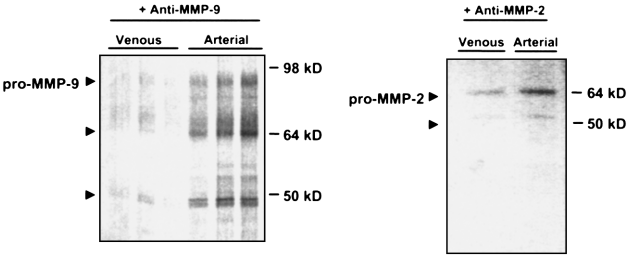

Figure 2. Arterial hemodynamic conditions increase MMP-9 and MMP-2 de novo production by veins after 3 days of perfusion, as demonstrated by pulse-biosynthetic radiolabeling with [^{35}S]methionine for 6 hours. Immunoprecipitated gelatinases are revealed by autoradiography. Left, Identification of newly synthesized pro-MMP-9 and processed forms, indicated by arrows. Right, Identification of radiolabeled pro-MMP-2 and active MMP-2.

were normalized to values obtained from matched segments perfused under venous conditions. The letter n always represents the number of independent experiments in which pairs of matched vein segments were investigated. Means of normalized levels were compared by 2-tailed Student *t* test, with a value of $P \leq 0.05$ accepted as significant.

Results

Vein Graft Morphology and Viability

Perfused and fresh vein segments showed similar typical saphenous vein morphology, including areas of intimal thickening, a tunica media with longitudinally and circumferentially organized SMCs, and a loosely organized adventitia with multiple vasa vasora. Regardless of hemodynamic conditions, or general morphology, endothelial immunostaining (not shown) demonstrated variable (25% to 90%) luminal coverage, similar to that previously reported for vein grafts in situ.[28,29]

Gelatinolytic bands secreted by veins corresponded to latent (pro) MMP-9, pro-MMP-2, and active MMP-2, as previously found for human saphenous vein SMCs[23] and as confirmed by Western blotting. Phorbol myristate acetate (25 nmol/L) or 10 ng/mL tumor necrosis factor-α (R&D Systems) stimulated pro-MMP-9 tissue levels in human saphenous vein maintained in perfusion systems for 3 days and left the MMP-2 level unchanged (see Figure I, which can be accessed online at http://atvb.ahajournals.org), as previously found in cultured human saphenous vein SMCs,[23] indicating that veins remained viable with intact gelatinase metabolic pathways for up to 5 days in the ex vivo system.

Arterial Hemodynamics Affect MMP-9 and MMP-2 Levels and Distribution

Comparison of MMP-9 and MMP-2 tissue levels in veins maintained in different hemodynamic conditions showed that although there was no statistically significant difference between static and venous conditions (pro-MMP-9, $P=0.95$; pro-MMP-2, $P=0.60$; and active MMP-2, $P=0.20$; n=4 independent experiments), levels were significantly affected in arterial conditions. After 3 days of perfusion, arterially perfused vein segments contained 50±8% less pro-MMP-9 ($P=0.01$), 44±6% less pro-MMP-2 ($P=0.005$), and 35±14% less active MMP-2 ($P=0.08$) than their venously perfused counterparts (n=4), by gelatin zymography (Figure 1). Lower levels of MMP-9 and MMP-2 were also detected in the culture media (not shown). To examine whether the decreased levels of zymogens are due to decreased synthesis, we investigated the effects of hemodynamic conditions on de novo MMP production by metabolic labeling of matching perfused vein segments. Interestingly, immunoprecipitation of MMP-9 and MMP-2 showed that arterial perfusion actually increased the level of newly synthesized MMP-9 and MMP-2 (Figure 2), suggesting that the apparent decrease in zymogen levels may be due to an increased posttranslational processing, eventually leading to MMP protein degradation.[30] Immunoprecipitation and immunostaining support a different distribution of MMP-9 and MMP-2. Newly synthesized MMP-9 was exclusively detected in the culture media, suggesting rapid secretion and low tissue retention, also supported by low levels of tissue specimen immunostaining (Figure 3). In contrast, radiolabeled MMP-2 was detected primarily in tissue extracts, supporting the higher retention in the extracellular space, reflected by the intensely positive tissue immunostaining. MMP-2 was detected extracellularly and intracellularly, whereas MMP-9 staining was mostly intracellular. Although no overall quantitative differences in MMP immunostaining were detected in frozen tissue sections of arterially or venously perfused vein segments, in the arterially perfused vein sections, staining for both MMPs appeared less intense in the inner layers and more intense in the outer layers of the wall (Figure 3). Interestingly, increased gelatinolytic activity and increased cell proliferation were detected in the outer layers, suggesting the preferential early remodeling of outer vein wall layers of the graft and supporting the contribution of MMP activity.

Cellular Proliferation

Cellular proliferation, identified by BrdU nuclear incorporation, occurred predominantly in the adventitia and outer media after 3 days of perfusion, with only occasional inner medial or intimal proliferating cells. The proliferation index

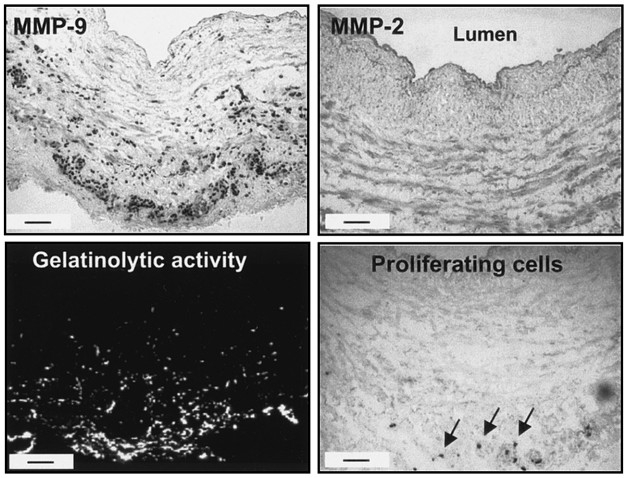

Figure 3. Detection of MMP-9 and MMP-2 by immunocytochemistry in arterially perfused veins suggests increased protein expression in the outer layers of vein wall. MMP-9 immunostaining showed high intracellular staining likely due to stimulated MMP-9 production. Less extracellular staining is likely due to poor retention of MMP-9 in the extracellular space. MMP-2 immunostaining was detected in the extracellular and intracellular compartments. Similar to MMP-9 and MMP-2, gelatinolytic activity (indicated by in situ zymography) and proliferating cells (assessed by immunodetection of BrdU incorporation, some indicated by arrows) also were detected predominantly in the adventitia and outer media of the vein wall. Bar=100 μm.

of arterially perfused vein segments was higher than that of venously perfused vein segments, with values between 200% and 750%, with a mean of 288±127% (P=0.07, n=9 microscopic fields per vein segment) in 4 of 6 matched pairs, whereas in 2 other experiments, there was no difference (not shown). Staining of serial sections with an endothelial cell marker identified some of these proliferating cells as endothelial cells of the vasa vasorum (not shown), whereas others were presumed to be SMCs or myofibroblasts.

Potential Mechanisms of Gelatinase Activation

On the basis of our results showing that arterial conditions actually stimulate MMP production, we further investigated potential activation pathways that could explain the decreased zymogen levels. First, we analyzed the expression of MT-MMP-1, an activator of MMP-2 expressed by human vascular cells,[31] and found that perfusion conditions did not affect its levels (see Figure II, which can be accessed online at http://atvb.ahajournals.org), as measured by Western blotting.

We then investigated the possible regulation of MMP processing due to the redox environment. We showed previously that activation of gelatinase zymogens produced by human SMCs can be triggered by interaction with reactive oxygen species.[32] Shear stress and pulsatile stretch were reported to increase the release of such species by isolated vascular cells.[33,34] Indeed, when we compared intact vein segments, we found that compared with venous conditions, arterial perfusion increased the superoxide levels by 240% to 580% (average increase 350±100%, P<0.05, n=4), as measured by chemiluminescence (Figure 4). The addition of Cu/Zn SOD eliminated the counts, indicating that chemiluminescence was solely due to superoxide. Furthermore, because the exogenous Cu/Zn SOD does not cross the cell plasma membrane, the result suggests that all superoxide detected was present in the extracellular space. Superoxide detected in the extracellular space may have been generated extracellularly or may have come from intracellular sources. When we examined semiquantitatively the intracellular levels of superoxide with the use of dihydroethidium staining (not shown), we found no differences between arterial and venous conditions.

Mechanisms of Increased Superoxide Levels in Arterially Perfused Vein Grafts

To sort out potential pathways of increased superoxide levels under arterial conditions, we tested the effects of several inhibitors (Figure 4). We found that the addition of diphenyleneiodonium, a flavoprotein inhibitor, completely inhibited chemiluminescence, ruling out nonflavoproteins, such as xanthine/xanthine oxidase. Although we cannot rule out the contribution of other flavoprotein-dependent enzymes, such as NO synthase, which under certain circumstances may produce superoxide, the vascular NAD(P)H oxidase is the most likely major source of superoxide production[35] in the vein wall. Treatment of veins with diethyldithiocarbamate, a copper chelator inhibitor of SOD, eliminated the difference between superoxide levels in venously and arterially perfused veins (782±190% versus 786±20%, relative to matched nontreated venously perfused controls; n=4; P=0.98; Figure 4), suggesting that increased superoxide production in arterially perfused veins is due to decreased scavenging by SOD.

When we further investigated this possibility, we found that vein tissue content of ecSOD was greatly reduced in arterially perfused compared with venously perfused tissue (Figure 5). In contrast, hemodynamic conditions did not

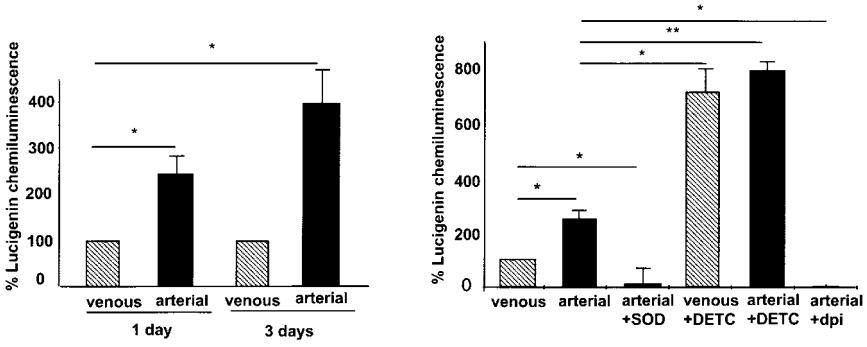

Figure 4. Arterial hemodynamic conditions increase superoxide levels released by vein rings perfused ex vivo, as assessed by 5 μmol/L lucigenin chemiluminescence counts. Counts have been normalized to matching venous conditions. Left, Chemiluminescence from matched vein pairs perfused for 1 and 3 days. Right, Effects of various treatments on superoxide released from perfused vein segments (1 day): Cu/Zn SOD (250 U/mL), diethyldithiocarbamate (DETC, 10 mmol/L), or diphenyleneiodonium (dpi, 10 μmol/L). See text for details. Results are expressed as mean±SEM (n=4 independent experiments for each condition). *P<0.05; **P<0.001.

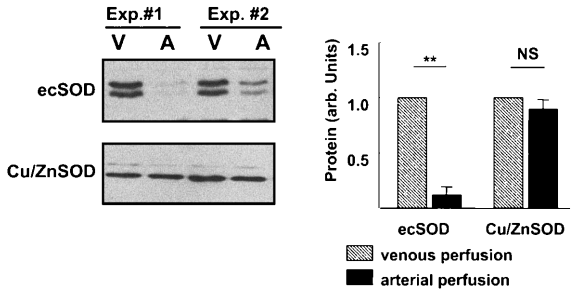

Figure 5. Hemodynamic conditions affect levels of vein graft SOD, as indicated by Western blotting. Left, Representative blot of ecSOD and cytosolic (Cu/Zn) SOD from 2 individual experiments (Exp.). V indicates venous perfusion; A, arterial perfusion. Right, Levels (normalized to venous conditions) of ecSOD and Cu/Zn SOD as determined by densitometric analysis. Results are expressed as mean±SEM (n=4 for ecSOD, n=3 for Cu/Zn SOD). $^{**}P<0.001$.

affect Cu/Zn SOD levels, suggesting that arterial hemodynamic conditions specifically affect superoxide scavenging in the extracellular compartment. Dihydroethidium staining, which primarily reflects intracellular superoxide, supported this hypothesis. We detected similar levels and patterns of staining in venously and arterially perfused veins (not shown), further supporting the concept that differences in total superoxide levels are due to different levels of extracellular superoxide.

Discussion

In the present investigation, we specifically questioned the effect of arterial hemodynamic conditions on human vein graft MMPs. Because all the veins used in the present study had been prepared for grafting, they were predisposed to intimal hyperplasia.[15] Strict inclusion criteria on the specimens obtained from the bypass surgery limited the number of experiments that we were able to carry out, but at the same time, they minimized variances. Porter et al[21] demonstrated previously that arterial shear stress inhibits the development of intimal hyperplasia in cultured vein pieces, but the additional impact of increased pressure and pulsatility has not been assessed. Furthermore, in their system, vein pieces were cut open and encased in silicon, disrupting the natural vein structure and consequently altering the forces experienced by vascular cells. Our perfusion system allowed us to create aggregate conditions similar to the venous or arterial hemodynamic environments and to study their effects on freshly harvested intact vein segments surgically prepared for grafting.

We found that MMP-9 and MMP-2 levels under venous perfusion conditions were similar to those in static conditions, previously used for study of vein intimal hyperplasia.[36] Contrary to what we expected, our findings showed that MMP zymogen levels were decreased under arterial perfusion conditions. We also examined effects of different perfusion conditions on the levels of major endogenous inhibitors of gelatinases (tissue inhibitor of metalloproteinase-1 or -2, data not shown), but we found no significant differences. Thus, hemodynamic conditions seem to influence the vein-degradative capacity through direct effects on MMPs. MMP-9 and MMP-2 distribution appeared decreased in the intima and inner media of the arterially perfused vein grafts, areas affected by changes during the formation of intimal hyperplasia. We suggest that this mechanism could work to limit the development of intimal hyperplasia triggered by surgical preparative injury in grafts experiencing normal arterial hemodynamics. This scenario is supported by the clinical observation that vein grafts with normal arterial flow rates develop less intimal hyperplasia[20] and have higher patency rates[37] than either technically compromised grafts or grafts with poor distal runoff, both subjected to lower flow rates.

We found that although arterial conditions stimulate MMP de novo synthesis, tissue levels under the same conditions are decreased. Among the potential explanations that we considered was an increased mass transfer, or a "washout" of tissue MMPs at high arterial perfusion pressure and flow rates. However, we found no differences in MMP-9 or MMP-2 levels in the perfusates of venously or arterially perfused tissues (data not shown). We then considered potential pathways for increased posttranslational proteolytic processing of MMP zymogens, which, once initiated, results in subsequent MMP degradation. We found that arterial conditions increased the superoxide levels 3- to 4-fold, supporting the contribution of increased redox stress under arterial conditions to rapid processing and inactivation of gelatinase zymogens. Taken together, the results from the biochemical assays and dihydroethidium staining indicate that hemodynamic conditions specifically affected the levels of extracellular superoxide. Furthermore, in our experiments with whole vein segments, the effect seemed to be due to modulation of the superoxide-scavenging capacity of veins rather than to increased superoxide production, as previously demonstrated in isolated vascular cells with cyclic stretch[33] and oscillatory shear.[34] Differences may be due to the fact that previous studies investigated expression in isolated cells. Although the source of extracellular superoxide cannot be definitively demonstrated, positive dihydroethidium staining indicated that superoxide was present in cells throughout the vein wall. The cell type was not specifically investigated.

We found that arterially perfused veins had substantially decreased tissue levels of ecSOD, but not of cytosolic Cu/Zn SOD, which was consistent with previous observations in endothelial cells. To our knowledge, regulation of ecSOD expression directly by mechanical forces has not been described previously. Although the ecSOD activity was not directly measured, inasmuch as activity levels characteristically parallel protein levels,[38] we suggest that its scavenging activity was decreased in veins under arterial hemodynamic conditions. Besides triggering the processing of MMP zymogens secreted by vascular cells in the extracellular space, decreased superoxide scavenging may increase the oxidative modification of other extracellular proteins, such as inspissated plasma lipoproteins, predisposing grafts to the formation of atherosclerotic lesions.

In the ex vivo conditions used in the present experiments, early cell proliferation occurred primarily in the outer layers of the vein wall. Differences found in the majority of specimens further support the contribution of hemodynamics, because in our experiments, comparisons were made in matched segments derived from the same vein, which minimized the contribution of surgical prepar-

ative injury, a major stimulus.[14] Preferential localization of cell proliferation in the outer layers of vein specimens suggests that in conditions of adequate perfusion of grafts, the compensatory vein wall thickening, necessary to normalize wall stress, may take place through adventitial and medial wall hyperplasia instead of intimal hyperplasia. It is also possible, as suggested previously, that proliferating myofibroblasts migrate into the intima of vein grafts, contributing to intimal hyperplasia[39] at later time points. In the present study, we did not perform morphometric analysis of vein grafts, because noticeable changes are likely to occur at times >3 days and because specimen fixation is incompatible with the biochemical functional analysis we performed. Thus, long-term effects of arterial hemodynamic conditions remain to be demonstrated.

Therefore, we identified likely components of early remodeling that are influenced by arterial hemodynamics: upregulation of MMP synthesis, increased MMP zymogen processing, and cellular proliferation. All these processes may be mediated via effects on the redox state of the vessel wall, as suggested by our present observations and by previous studies showing the many regulatory functions of reactive oxygen species. Detection of increased superoxide levels in vein grafts, which are likely due to diminished scavenging by ecSOD, may provide insight into future therapeutic strategies for vein graft disease. We previously proposed the use of antioxidants to limit the degrading activity of vascular MMPs.[40] Antioxidant treatment has already been associated with reduced restenosis and remodeling in patients who undergo angioplasty.[41] Similar treatment, or direct vein graft gene therapy, may result in reduced intimal hyperplasia and atherosclerosis, lowering the incidence of recurrent ischemia in those undergoing bypass grafting.

Acknowledgments

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References

1. Cox JL, Chiasson DA, Gotlieb AI. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences between veins and arteries. *Prog Cardiovasc Dis*. 1991;34:45–68.
2. Davies MG, Hagen PO. Pathophysiology of vein graft failure: a review. *Eur J Vasc Endovasc Surg*. 1995;9:7–18.
3. Zwolak RM, Adams MC, Clowes AW. Kinetics of vein graft hyperplasia: association with tangential stress. *J Vasc Surg*. 1987;5:126–136.
4. Westerband A, Mills JL, Marek JM, Heimark RL, Hunter GC, Williams SK. Immunocytochemical determination of cell type and proliferation rate in human vein graft stenoses. *J Vasc Surg*. 1997;25:64–73.
5. Fuchs JC, Mitchener JS III, Hagen PO. Postoperative changes in autologous vein grafts. *Ann Surg*. 1978;188:1–15.
6. Southgate KM, Davies M, Booth RF, Newby AC. Involvement of extracellular-matrix-degrading metalloproteinases in rabbit aortic smooth-muscle cell proliferation. *Biochem J*. 1992;288:93–99.
7. Pauly RR, Passaniti A, Bilato C, Monticone R, Cheng L, Papadopoulos N, Gluzband YA, Smith L, Weinstein C, Lakatta EG, et al. Migration of cultured vascular smooth muscle cells through a basement membrane barrier requires type IV collagenase activity and is inhibited by cellular differentiation. *Circ Res*. 1994;75:41–54.
8. Bendeck MP, Zempo N, Clowes AW, Galardy RE, Reidy MA. Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Circ Res*. 1994;75:539–545.
9. Southgate KM, Fisher M, Banning AP, Thurston VJ, Baker AH, Fabunmi RP, Groves PH, Davies M, Newby AC. Upregulation of basementmembrane-degrading metalloproteinase secretion after balloon injury of pig carotid arteries. *Circ Res*. 1996;79:1177–1187.
10. Zempo N, Kenagy RD, Au YP, Bendeck M, Clowes MM, Reidy MA, Clowes AW. Matrix metalloproteinases of vascular wall cells are increased in balloon-injured rat carotid artery. *J Vasc Surg*. 1994;20:209–217.
11. Senior RM, Griffin GL, Fliszar CJ, Shapiro SD, Goldberg GI, Welgus HG. Human 92- and 72-kilodalton type IV collagenases are elastases. *J Biol Chem*. 1991;266:7870–7875.
12. George SJ, Zaltsman AB, Newby AC. Surgical preparative injury and neointima formation increase MMP-9 expression and MMP-2 activation in human saphenous vein. *Cardiovasc Res*. 1997;33:447–459.
13. Soyombo AA, Angelini GD, Bryan AJ, Newby AC. Surgical preparation induces injury and promotes smooth muscle cell proliferation in a culture of human saphenous vein. *Cardiovasc Res*. 1993;27:1961–1967.
14. Meng XP, Mavromatis K, Galis ZS. Mechanical stretching of human saphenous vein grafts induces expression and activation of matrix-degrading enzymes associated with vascular tissue injury and repair. *Exp Mol Pathol*. 1999;66:227–237.
15. Soyombo AA, Angelini GD, Newby AC. Neointima formation is promoted by surgical preparation and inhibited by cyclic nucleotides in human saphenous vein organ cultures. *J Thorac Cardiovasc Surg*. 1995;109:2–12.
16. George SJ, Johnson JL, Angelini GD, Newby AC, Baker AH. Adenovirus-mediated gene transfer of the human TIMP-1 gene inhibits smooth muscle cell migration and neointimal formation in human saphenous vein. *Hum Gene Ther*. 1998;9:867–877.
17. Cowan DB, Langille BL. Cellular and molecular biology of vascular remodeling. *Curr Opin Lipidol*. 1996;7:94–100.
18. Predel HG, Yang Z, von Segesser L, Turina M, Buhler FR, Luscher TF. Implications of pulsatile stretch on growth of saphenous vein and mammary artery smooth muscle. *Lancet*. 1992;340:878–879.
19. Schwartz LB, O'Donohoe MK, Purut CM, Mikat EM, Hagen PO, McCann RL. Myointimal thickening in experimental vein grafts is dependent on wall tension [see comments]. *J Vasc Surg*. 1992;15:176–186.
20. Dobrin PB, Littooy FN, Endean ED. Mechanical factors predisposing to intimal hyperplasia and medial thickening in autogenous vein grafts. *Surgery*. 1989;105:393–400.
21. Porter KE, Nydahl S, Dunlop P, Varty K, Thrush AJ, London NJ. The development of an in vitro flow model of human saphenous vein graft intimal hyperplasia. *Cardiovasc Res*. 1996;31:607–614.
22. Chesler NC, Conklin BS, Han HC, Ku DN. Simplified ex vivo culture techniques for porcine arteries. *J Vasc Invest*. 1998;4:123–127.
23. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Unemori EN, Lark MW, Amento E, Libby P. Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ Res*. 1994;75:181–189.
24. Galis ZS, Sukhova GK, Libby P. Microscopic localization of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue. *FASEB J*. 1995;9:974–980.
25. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest*. 1993;91:2546–2551.
26. Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA. Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem*. 1998;273:2015–2023.
27. Miller FJ Jr, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circ Res*. 1998;82:1298–1305.
28. Ramos JR, Berger K, Mansfield PB, Sauvage LR. Histologic fate and endothelial changes of distended and nondistended vein grafts. *Ann Surg*. 1976;183:205–228.
29. Adcock OT Jr, Adcock GL, Wheeler JR, Gregory RT, Snyder SO Jr, Gayle RG. Optimal techniques for harvesting and preparation of reversed autogenous vein grafts for use as arterial substitutes: a review. *Surgery*. 1984;96:886–894.

30. Woessner JF Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J*. 1991;5:2145–2154.
31. Rajavashisth TB, Liao JK, Galis ZS, Tripathi S, Laufs U, Tripathi J, Chai NN, Xu XP, Jovinge S, Shah PK, et al. Inflammatory cytokines and oxidized-low density lipoproteins increase endothelial cell expression of membrane type-matrix metalloproteinase-1. *J Biol Chem*. 1999;274: 11924–11929.
32. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro: implications for atherosclerotic plaque stability. *J Clin Invest*. 1996;98:2572–2579.
33. Hishikawa K, Oemar BS, Yang Z, Luscher TF. Pulsatile stretch stimulates superoxide production and activates nuclear factor-kappa B in human coronary smooth muscle. *Circ Res*. 1997;81:797–803.
34. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *Circ Res*. 1998;82:1094–1101.
35. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest*. 1997;100:2153–2157.
36. Soyombo AA, Angelini GD, Bryan AJ, Jasani B, Newby AC. Intimal proliferation in an organ culture of human saphenous vein. *Am J Pathol*. 1990;137:1401–1410.
37. Grondin CM, Meere C, Castonguay Y, Lepage G, Grondin P. Progressive and late obstruction of an aorto-coronary venous bypass graft. *Circulation*. 1971;43:698–702.
38. Fukai T, Galis ZS, Meng XP, Parthasarathy S, Harrison DG. Vascular expression of extracellular superoxide dismutase in atherosclerosis. *J Clin Invest*. 1998;101:2101–2111.
39. Shi Y, O'Brien JE Jr, Mannion JD, Morrison RC, Chung W, Fard A, Zalewski A. Remodeling of autologous saphenous vein grafts: the role of perivascular myofibroblasts. *Circulation*. 1997;95:2684–2693.
40. Galis Z, Asanuma K, Godin D, Meng X. N-Acetyl-cysteine decreases the matrix-degrading capacity of macrophage-derived foam cells: new target for antioxidant therapy? *Circulation*. 1998;97:2445–2454.
41. Cote G, Tardif JC, Lesperance J, Lambert J, Bourassa M, Bonan R, Gosselin G, Joyal M, Tanguay JF, Nattel S, et al. Effects of probucol on vascular remodeling after coronary angioplasty: Multivitamins and Protocol Study Group. *Circulation*. 1999;99:30–35.