Increased Neointimal Formation After Surgical Vein Grafting in a Murine Model of Type 2 Diabetes

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Background—Diabetes is an independent risk factor for the development of neointimal hyperplasia and subsequent vein graft failure after coronary or peripheral artery bypass grafting. We evaluate a new mouse model of surgical vein grafting to investigate the mechanisms of neointimal formation in the setting of type 2 diabetes.

Methods and Results—Surgical vein grafts were created by inserting vein segments from age-matched C57BL/KsJ wild-type mice into the infra-renal aorta of lepr<sup>db/db</sup> diabetic and C57BL/KsJ wild-type mice. Mice were euthanized after 4 weeks, and vein grafts were analyzed using morphometric and immunohistochemical techniques. A significant increase in neointimal formation was noted in lepr<sup>db/db</sup> mice (139±64 versus 109±62 mm<sup>2</sup>; P=0.008) after 4 weeks. This difference was mainly secondary to an increase in collagen formation within the lesion in the vein grafts from lepr<sup>db/db</sup> mice (0.53±0.4 versus 0.44±0.05; P<0.001), whereas only slight increases (P=not significant) in alpha actin-stained smooth muscle cells were noted in the lepr<sup>db/db</sup> mice.

Conclusion—We established a new physiologically relevant model of surgical vein grafting in mice. In this report, type 2 diabetes was associated with significant increase in extracellular matrix deposition in addition to increased smooth muscle cell deposition. This new model may allow mechanistic studies of cellular and molecular pathways of increased neointimal formation in the setting of diabetes. (Circulation. 2006;114[suppl I]:I-302–I-307.)

Key Words: coronary artery disease • diabetes • mouse • neointima • surgery

Coronary artery bypass surgery (CABG) using a left internal mammary artery and saphenous vein conduits remains the most common surgical revascularization strategy in patients with ischemic heart disease. Within 10 years, however, vein graft occlusive disease occurs in nearly half of the conduits, and patients with diabetes are particularly at risk.1

Increased neointimal formation is a pathologic hallmark of occlusive vein graft disease not only after CABG but also after peripheral vascular surgery.2 Initially, biomechanical stress may lead to cell death within newly arterialized veins.3 Smooth muscle cell (SMC) migration into the vascular intima with extracellular matrix (ECM) deposition then occurs, ultimately leading to increased neointimal formation.2,4

Diabetes plays an important role in the development and modulation of neointimal formation.5–8 The mechanism by which diabetes influences vascular responses remains unknown and poorly investigated. To date no animal models exist for the identification and understanding of the precise molecular and cellular pathways involved in the diabetic vein graft failure.9 Using syngeneic and mutant mice, we sought to establish a murine model that would allow us to study the potential impact of diabetes on vein graft neointimal formation.

Methods

Animal Model

Mice were purchased from Jackson Laboratory (Bar Harbor, Me). We used 2 types of mice in this experiment. Donor mice were male wild-type (WT) C57BL/KsJ (JAX strain 000662) and mutant heterozygotes BKS lepr<sup>db/+</sup> (JAX strain 000699). Recipients were male WT C57BL/KsJ (JAX strain 000662) and C57BL/KsJ lepr<sup>db/db</sup> (JAX strain 000642). Mice were allowed free access to food (Chow Purina diet 5053; Purina Mills, LLC) and water.

The mutant lepr<sup>db/db</sup> mouse is a leptin receptor-deficient mouse with many of the metabolic abnormalities noted in human obese diabetic subjects, such as obesity, hyperglycemia, elevated circulating leptin levels, and insulin resistance.10

Study Design

Two groups of mice were used, consisting of male age matched lepr<sup>db/db</sup> (n=10) and wild-type (WT) (n=14) mice. Donors were either lepr<sup>db/+</sup> C57BL/KsJ. Mice were subject to surgical allograft vein grafting as described below. The protocol was approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine, New York, NY, and all experimental procedures were consistent with the Guide for the Care and Use of Laboratory Animals.

Vein Graft Procedure

Microsurgical procedures were performed aseptically in a dedicated facility for rodent microsurgery. Isoflurane (Baxter, Deerfield, Ill)
was mixed in an automated vaporizer with room air in a regulated concentration, and was administered by way of a nose cone. A surgical stereomicroscope (Zeiss, Germany), with variable magnification (20× to 50×) was used for all procedures.

In donors, the inferior vena cava was harvested through a right lateral chest flap. It was identified and dissected free from the area of its connection with the right atrium and liberated from fatty adhesions down to the level of the diaphragm. The vena cava was then transected at its diaphragmatic and atrial extremities, and placed in a solution composed of fresh autologous blood and sterile Medium (Invitrogen Life Technologies, Inc, Carlsbad, Calif).

In recipients, through a midline incision, the infrarenal abdominal aorta was fully isolated and mobilized from the adjacent vena cava, all side branches were cauterized (Cautery; Aaron Medical, St. Petersburg, Fla). The mouse was heparinized by an intra venous injection of heparin 100 IU/kg body weight (Heparin Lock Flush; Solution USP, Laboratories, Chicago, Ill). In the leprdb/db mice we used one-quarter of the 100 IU/kg body weight used in the WT. Occlusion of the aorta was obtained with 2 clamps (Micro-vascular, size B-1; Fine Science Tools, Foster City, Calif), proximally below the renal arteries and distally as close as possible to the iliac bifurcation. The aorta was transected and flushed with saline (Figure 1A). The segment of donor vein was grafted between the 2 ends by end-to-end anastomosis with 11-0 Ethilon (Bear Micro Surgery; ARO Surgical, Newport Beach, Calif) interrupted sutures (Figure 1B and 1C). The distal clamp was then removed to allow retrograde filling and closure of any defect with additional sutures, and finally the proximal clamp was released (Figure 1D). Figure 2 shows a completed in vivo interposition graft. Pulsatile flow and hind limb re-coloring subjectively confirmed graft patency. Protamine sulfate (Eli Lilly, Indianapolis, Ind) was injected intravenously in a 1:1 ratio to heparin. Closure was performed in 2 layers with 3-0 Vicryl sutures (Ethicon, Johnson & Johnson, Piscataway, NJ). Animals were placed under a heating lamp (Reflector Infrared, Philips, the Netherlands) until normal activity was regained. Postoperative analgesia was provided by Buprenex (Rickitt Benckiser Pharmaceuticals Inc, Richmond, Va) at 0.05 mg/kg intraperitoneal twice daily during 3 days.

Biochemical Measurements and Euthanization
Preoperative metabolic data were obtained in all animal groups. At the time of euthanization, all mice were weighed and leprdb/db mice were fasted for 6 hours for tail vein blood samples for measurements of plasma glucose and insulin levels. Vein grafts were harvested en bloc with a cuff (or short segment) of native aorta after 1 month. This time point is in line with previous studies demonstrating a steady state in neointimal hyperplasia after 1 month.11,12 For this, mice were premedicated with high-rate isoflurane and perfusion fixed with fresh 4% para-formaldehyde in phosphate-buffered saline. Specimens were labeled and paraffin-embedded in a core pathology facility.

Histology
Combined Masson elastin (CME)-stained cross-sections were used for morphometric assessment using a Nikon Eclipse E400 (Nikon Instruments Inc) microscope equipped with a SPOT 3-shot Insight...
QE camera (Diagnostic Instruments, Stirling Heights, Mich). Images were cropped with the SPOT software (version 3.5.7 for Windows; Diagnostic Instruments, Stirling Heights, Mich) and analyzed using Bersoft Image Measurement 2.01 software (Bersoft Inc, Ontario, Calif) by independent outside researchers in a blinded manner. Eight to 10 sections from each animal within the vein graft were identified and used for morphometric assessment. The neointimal area was determined by subtracting the area of the lumen from the area encircled by the inner border of the adventitial collagenoid reaction.14 ECM and SMC ratio calculations were performed by morphometric analysis of the lesion content in green (collagen) on CME, and red (SMC) content on alpha actin.13 A ratio of ECM over adventitial and intimal tissue reaction was defined in a blinded manner and used for ECM morphometric assessment. Immunohis-tochemical staining was performed on every fifth slide with the following stains: alpha actin, ICAM, MOMA 2, and ki67.

Statistics
All quantitative measurements were performed in a blinded manner. Morphometric data are given as mean±standard deviation. All calculations were performed on SPSS statistical program (Version 12; SPSS Inc, Chicago, Ill). Comparison between control and treatment groups was made with a 2-tailed t test, and differences were considered significant when P<0.05.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results
Surgical Outcomes
From December 2003 to April 2004, a total of 24 surviving animals were included (n=10 lepr id and n=14 WT). Early in the series in the WT group, 2 mice died early (days) due to graft thrombosis, after prolonged aortic cross-clamp time (>50 minutes). In the lep id group, 3 mice died perioperatively from surgical bleeding at the anastomotic site.

The aorta was clamped for a mean duration of 34±5 minutes in all animals. Duration for surgical dissection of the abdominal aorta was significantly increased in the lep id group (46±3 versus 30±2 minutes; P<0.001) mainly because of the significant difference in body weight and composition, these mice being obese. Animals were euthanized after a mean duration of 30±8 days. When compared with age-matched litter mate controls, lep id recipient mice were obese (45±3 versus 26±4 grams; P<0.001), hyperglycemic (443±82 versus 136±11 mg/dL; P<0.001), and hyperinsulinemic (4.7±2.7 versus 0.7±0.2 ng/mL; P<0.001).

Histological Assessment
Histological examination of all cross-sections through the infrarenal vein graft was performed on CME-stained sections. A complete and circumferentially viable vein graft with a patent lumen was identified in all. Figure 3 shows representative sections on CME stain of a normal WT inferior vena cava (Figure 3A) in low (4×) magnification; Figure 3B depicts a low magnification (4×) vein graft in the WT; and (Figure 3D) in the lep id group. Figure 3C and 3E are higher magnifications (20×) of WT and lep id areas of interest. On higher magnification, the neointima is readily defined by its inner limits composed of the lumen of the vessel, and its outer limits, which was defined as an adventitial collagenoid reaction in both groups. These landmarks were used to quantify neointimal formation and ECM deposition in a blinded manner. On microscopic inspection, on all CME slides from both groups, a heterogeneously distributed neo-intimal formation was seen within the entire graft circumference, this heterogeneity was similar in all animals (data not shown).

On quantitative assessment, neointimal formation was significantly increased in the lep id group (139±64 versus 109±62 mm²; P=0.008) (Figure 3F). Green-stained collagen accumulation was seen on CME staining, leading to the creation of an external layer, similar to an adventitial tissue layer seen in arteries. This reaction and accumulation was quantified and seen to be significantly increased in the lep id group (ECM/lesion and adventitial ratio, 0.53±0.4 versus 0.44±0.05; P<0.001) (Figure 3G). Alpha actin stains demonstrated a smooth muscle cell phenotype as a predominant cell type within the neointimal formation, and showed relative similar alpha actin expression within this lesion (Figure 4). The lep id group had a similar alpha actin content when compared with the WT (2.5±0.05 versus 2.9±0.1; P=0.22) (Figure 4E). ICAM staining revealed a continuous unicellular layer of functional endothelial cells in both groups. MOMA 2 specific macrophage staining demonstrated the absence of any significant macrophage cell accumulation in the neointimal area of both control WT and lep id animals (data not shown). Ki67 labeling was performed on 5 representative sections from each group. The ratio of nuclei stained with Ki67 over the lesion area was similar in both groups.

Discussion
Murine in vivo models are appealing because of their well-defined genetic background, and the possibility of using syngeneic “knockout” and mutant mice producing a large known variety of metabolic settings. To date few relevant murine models that permit mechanistic study of neointimal formation in different metabolic disorders exist.14–16 The first aim of this experiment was intended at creating a new model to provide the possibility to assess neointimal formation after surgical vein grafting in a clinically and physiologically relevant manner. The second part of the experiment included the application of this technique to a murine model of type 2 diabetes, the lep id strain.19 We demonstrated the feasibility of the model in WT mice and then demonstrated a significant increase in neointimal formation after 4 weeks in the lep id mouse.

A murine model of vein graft disease ideally reproduces flow and pressure characteristics typical of the clinical setting. Perhaps the most commonly used procedure was first reported by Zou et al.17 This is a surgical interposition of a segment of vena cava in the common carotid artery by means of 2 cuff anastomosis. Another technique described by Zhang et al16 uses an inferior vena cava graft, which is surgically anastomosed with 2 end-to-side anastomosis to the common carotid artery. Recently, Diao et al15 performed an end-to-end anastomosis in the infrarenal abdominal aorta using autolo-gous internal jugular veins. Our choice of inferior vena cava (IVC) interposition in the aorta was to further simplify technical and reproducibility issues related to murine vein graft models. In addition, we wanted to establish a model in
Figure 3. Representative photomicrograph (4×) of combined Masson elastin-stained normal vein (A), vein graft sections control C57BKS (B), and Lepr<sup>db/db</sup> (D) mice. C and E, High-magnification (×20) areas of regions enclosed by a box (B and D). Morphometric measurements revealed significant differences in neointimal formation (F) and collagen content (G) between the 2 groups of mice.
which a hemodynamic steady state is achieved by subjecting a vein graft to a continuous high pressure arterial flow setting. This model offers the advantage of being independent and solely relying on cellular and molecular responses to set off and entertain neo-intimal formation.

From our results, it appears that SMC proliferation, leading to a multilayered neointima, in addition to ECM deposition both contribute to significant neointimal formation. In our series, when comparing lesion areas in WT and lepr\(^{db/db}\) mice, quantitative assessment demonstrated a trend in increase in SMC accumulation accompanied by a significant increase in ECM deposition. These data suggest that ECM deposition, in addition to relative SMC proliferation, may be an important factor contributing to neointimal formation in the lepr\(^{db/db}\) mouse. Renal studies on lepr\(^{db/db}\) mice revealed a link between high circulating leptin levels, increased TGF-\(\beta\), and glomerulosclerosis. Increased activity of the TGF-\(\beta\) system in the lepr\(^{db/db}\) mouse may be one of the mechanisms leading to increased neo-intimal formation, but may also play an important role leading to neo-intimal formation in the WT as seen in our series and in others. In the lepr\(^{db/db}\) mouse, the db/db mutation results in defective leptin signaling in the hypothalamus, thereby increasing appetite and food intake, which leads to a secondary metabolic syndrome. Although leptin

Figure 4. Representative photomicrographs (4\(\times\)) of alpha actin smooth muscle cell stain of vein graft sections and morphometric measurements from control C57BKS (A) and Lepr\(^{db/db}\) (C) mice. B and D, High-magnification (\(\times10\)) areas of regions enclosed by a box (A and C).
receptor mRNA has been detected in the periphery, the role of leptin outside of the central nervous system is unclear. It is possible that the effect on vein graft stenosis is caused by a combination of factors including diabetes and altered leptin signaling in the \textit{lepr} \textit{db/db} mouse. Interestingly, when Stephenson et al.\textsuperscript{19} investigated catheter femoral arterial injury in \textit{lepr} \textit{db/db} mice, the absence of vascular response in the \textit{lepr} \textit{db/db} mice was the main finding. Our results are in contradiction to both series; however, because we are studying arterIALIZATION of vein grafts, this may be in opposition to arterial injury as the underlying vascular response is different. Whereas with surgical vein grafting, it is not only the one-time pressure-related stress that initiates a chain of events, but more importantly a constant and relentless pulsatile mechanical stretch the vein graft is subjected to which will contribute to perpetuating vascular responses (SMC proliferation and ECM deposition). Although the absence of leptin receptors in the \textit{lepr} \textit{db/db} might have a role in SMC proliferation, we speculate that increased TGF-\(\beta\) activity may lead to increased ECM deposition in this setting.

Limitations

Like all animal models, ours does not exactly replicate the clinical flow characteristics of aorto-coronary bypass and therefore may not be directly reflective of the human setting. However, we believe murine models provide the best scenario for study of this disease, because of their simplicity and ability to genetically manipulate mice as opposed to large animal models. Unlike other workers, we have used an allograft rather than an autograft model. The high variability of neointimal deposition within the vein grafts is probably secondary to different flow patterns within the graft. This variability was present in all examined specimens to similar degrees and are unlikely to have impacted the overall findings significantly. Our histological findings, however, did not differ substantially from that in allograft models and because similar immunological burden was present in both the controls and the diabetic mice, we do not believe our findings can be explained by immunological reaction.

Conclusion

We have described a new murine model to investigate neointimal formation in an experimental diabetic setting. We demonstrated a significant increase of neointimal formation in the \textit{lepr} \textit{db/db} mouse and conclude that this is mainly caused by ECM deposition in addition to SMC proliferation. This new model should allow further mechanistic studies and may potentially provide preventive strategies against vein graft failure in diabetic patients after bypass surgery.

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Disclosures

None.

References

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