Lepr\textsuperscript{db} Diabetic Mouse Bone Marrow Cells Inhibit Skin Wound Vascularization but Promote Wound Healing

Vesna Stepanovic,* Ola Awad,* Chunhua Jiao, Martine Dunnwald, Gina C. Schatteman

Abstract—Bone marrow stem cells participate in tissue repair processes and may have roles in skin wound repair. Diabetes is characterized by delayed and poor wound healing, and type 1 diabetes seems to lead to stem cell dysfunction. Hence, stem cell dysfunction could contribute to poor healing, and stem cell–based therapies may be efficacious in diabetic wounds. We investigated the potential of exogenous stem cells to promote skin healing and possible effects of type 2 diabetes on stem cell function. Mouse bone marrow cells from nondiabetic and diabetic mice were enriched for putative stem cells and injected under skin wounds of nondiabetic or type 2 diabetic Lepr\textsuperscript{db} mice. Using histology and morphometry, vascularization and healing in treated and untreated mice were analyzed. We anticipated a correlation between improved wound healing and vascularization, because therapies that increase tissue vascularization tend to enhance wound healing. Our data indicate that exogenous nondiabetic bone marrow–derived cells increase vascularization and improve wound healing in Lepr\textsuperscript{db} mice but have little effect on nondiabetic controls. In contrast, Lepr\textsuperscript{db}-derived marrow cells inhibit vascularization but promote wound healing in Lepr\textsuperscript{db} mice. Thus, adult stem cell function may be impaired by type 2 diabetes; the ability to promote vascularization and wound healing are distinct functions of bone marrow cells; and neovascularization and wound healing may not be tightly coupled. Additionally, we observed little incorporation of injected cells into wound structures, suggesting that improved healing is mediated through mechanisms other than direct differentiation and incorporation of the cells. (Circ Res. 2003;92:88-96.)

Key Words: bone marrow \textbullet{} stem cells \textbullet{} angiogenesis \textbullet{} wound healing \textbullet{} type 2 diabetes

A subset of bone marrow–derived cells is believed to function as adult stem cells capable of differentiating into a variety of cell types, including endothelial cells (ECs). Marrow–derived hematopoietic progenitor cells expressing the cell surface antigen sca-1 (Ly-6A/E) in the mouse and CD34 in humans seem to be enriched for these stem cells.\textsuperscript{1}-\textsuperscript{6} At least 2 pieces of evidence suggest that marrow–derived stem/progenitor cells are dysfunctional in type 1 diabetes. Whereexogenous CD34\textsuperscript{+} cells from the blood of nondiabetic humans had no effect on the restoration of blood flow to an ischemic limb in nondiabetic mice, the same cells profoundly accelerated blood flow restoration in diabetic mice.\textsuperscript{7} Additionally, cultured CD34\textsuperscript{+} blood cells derived from type 1 diabetic patients produced fewer ECs per milliliter of blood than did cells from nondiabetic controls.\textsuperscript{7} Significantly, there was no difference in the number of ECs produced by cells derived from type 2 diabetic patients compared with controls, suggesting that stem/progenitor cell function may differ in type 1 and type 2 diabetes. Poor skin wound healing associated with diabetes is thought to result in part from impaired neovascularization, and delayed wound healing can lead to nonhealing ulcers.\textsuperscript{8}-\textsuperscript{12} Therapies that increase vascularization tend to enhance wound healing, suggesting that treatments that improve neovascularization could have important clinical applications.\textsuperscript{8}-\textsuperscript{12} Recently, we examined the potential of human CD34\textsuperscript{+} cells to increase vascularization and improve skin wound healing in a nude mouse model of type 1 diabetes.\textsuperscript{13} We observed a marked increase in vascularization after CD34\textsuperscript{+} cell injection and little effect on wound healing (which is not dramatically impaired in these mice). Because marrow–derived EC progenitor function may differ in type 1 and type 2 diabetes, it was unclear if a similar effect would be observed in a mouse model of type 2 diabetes. To examine this issue, we investigated the effects of mouse hematopoietic progenitor cells (mHPCs), which are enriched in Sca-1\textsuperscript{+} cells, on vascularization and wound healing of skin wounds in C57Bl/6 and Lepr\textsuperscript{db} mice. Lepr\textsuperscript{db} mice lack functional leptin receptors, become obese shortly after birth, and are insulin resistant and hyperglycemic as adults.\textsuperscript{14,15} Wound healing is impaired in these mice, although angiogenesis is not significantly affected.\textsuperscript{14,15} Wound healing is promoted by marrow–derived cells in nondiabetic controls, but the effect is less pronounced in type 2 diabetic mice. In contrast, marrow–derived cells from diabetic mice promote wound healing, suggesting that adult stem cell function is impaired by type 2 diabetes. Therefore, we investigated the potential of adult stem cells to promote skin wound repair in a mouse model of type 2 diabetes.

Materials and Methods

Wounding

Male C57Bl/6 mice served as nondiabetic controls, and male congenic Lepr\textsuperscript{db} (B6.Cg-m\textsuperscript{-1}Lepr\textsuperscript{+}) served as type 2 diabetic mice (8 to 10 weeks) (Jackson Laboratories, Bar Harbor, Maine). Mice were bred and maintained in the animal care facility of the University of Iowa. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee. Vascularization and healing in treated and untreated mice were analyzed. We anticipated a correlation between improved wound healing and vascularization, because therapies that increase tissue vascularization tend to enhance wound healing.
were anesthetized by intraperitoneal injection of 90 μg ketamine plus 10 μg xylazine/g, and their back skin was depilated with Nair and cleaned with povidone iodine. Using a sterile 6-mm biopsy punch, 2 bilateral full-thickness skin wounds were created on the dorsorotral back skin without injuring the underlying muscle. Wounds were separated by a minimum of 6 mm of uninjured skin. Three days later, mice were again anesthetized, and 2.5x10^3 freshly isolated bone marrow cells were collected from mHPCs in 25 μL 0.9% NaCl from either C57Bl/6 mice or male Lepr^db mice was injected under each wound. Controls received 25 μL 0.9% NaCl. To examine the incorporation of mHPCs in the wound vessels, wounds were injected as above with mHPCs derived from EGFP transgenic [TgN(ACTbEGFP)1Osb] mice. For all mice, both wounds were injected with the same substance to avoid the possibility that injected cells could have systemic effects or could migrate or secrete substances into the contralateral wound. Procedures were performed according to University of Iowa IACUC guidelines.

Isolation of mHPCs
Mice were injected intraperitoneally with a lethal dose (150 mg/kg) of sodium pentobarbital. Bone marrow cells were collected from femurs and tibias as described and enriched for mHPCs using Spin Sep according to manufacturer’s instructions (StemCell Technologies). This is a negative selection process that depletes marrow cells of cells expressing the lineage markers CD5, CD45R, CD11b, myeloid differentiation antigen, TER119, and a neutrophil antigen recognized by antibody 7-4. To determine the purity of the enriched cells, cells were resuspended at 1x10^6 cells/mL in PBS containing 5% rat serum. After 10 minutes, R-PE rat anti-mouse Ly-6A/E (Sca-1) (BD PharMingen) was added to 1 μg/mL and incubated for 30 minutes on ice, washed twice, resuspended in 500 μL PBS and overlaid onto 500 μL FCS, washed, fixed in 4% paraformaldehyde, and analyzed by FACS. Just before analysis, cells were stained with 1 μg/mL propidium iodide to label all nuclei.

Histological Procedures
Thirteen or 14 days after wounding, mice were anesthetized and depilated. The next day they were lethally injected with sodium pentobarbital as above. Wound beds and underlying muscle surrounded by a margin of normal skin were harvested, fixed 4 hours in 100% methanol, processed through 100% ethanol and xylenes, and paraffin embedded. Except where noted, 8 wounds (1 to 2 from 4 to 6 different mice) in each group were serially sectioned (7 μm) perpendicular to the wound surface rostral to caudally. Untreated control and day 0 wound analyses were performed on 6 C57Bl/6J and 6 Lepr^db mice. For day 0 tissue, mice were anesthetized, the skin was punched, and then the wound was harvested and fixed immediately.

Every 10th section throughout the entire wound bed was H&E-stained for wound analysis, and the adjacent section was immunolabeled with anti-CD31 (BD Pharmingen) to visualize blood vessels. Sections were cut, washed for 3 minutes at 37°C with 100 μg/mL proteinase K (BD Pharmingen) before 1 hour of incubation with 2.5 μg/mL anti-CD31 or rat IgG as control at 37°C in 0.75 μg/mL biotinylated anti-rat IgG and then 1:200 alkaline phosphatase-streptavidin complex (Vector) followed by visualization with Vector Red (Vector) and hematoxylin counterstaining. The number of sections analyzed ranged from 15 to 20 per wound, depending on the size of the wound. Representative sections from each wound were also stained with Masson’s trichrome to visualize collagen fibers in the wound bed.

To examine inflammatory responses in the wounds, wound tissue was harvested from 4 C57Bl/6 mice 5 days after wounding (2 days after treatment). Two mice had been injected with mHPCs and 2 with saline as above. Tissue was fixed in 4% paraformaldehyde, dehydrated, paraffin embedded, and serially sectioned. Every 10th section throughout the entire wound was H&E stained. Additional sections were immunolabeled to detect monocyte/macrophages. Sections were blocked and proteinase K was pretreated for immunolabeling as above, incubated 2 hours with 0.5 μg/mL anti-mouse ER-MP23 (BMA) that detects mouse macrophage galactose-specific lectin (MMGL), washed thrice in PBS, incubated 1 hour with 10 μg/mL Alexa 488 donkey anti-rat (Molecular Probes, Eugene, Oregon), washed again, and aqueous-mounted.

Wounds from C57Bl/6 and Lepr^db mice that were injected with EGFP transgenic mHPCs were harvested and fresh frozen. After sectioning and rapid fixation in 2% paraformaldehyde, sections were incubated with 5 μg/mL rabbit anti-green fluorescence protein (Novus Biologicals) and 1.25 μg/mL biotinylated Bandeira Simplicifolia isolecitin B, (Vector), followed by 1 hour with 10 μg/mL goat anti-rabbit Alexa 488 and 10 μg/mL Alexa 594 Streptavidin A (both from Molecular Probes), and then stained with DAPI to visualize nuclei.

Morphometry
To determine wound area, the wound periphery (epidermis and dermis) of H&E-stained sections was traced digitally from images (Nikon E600 microscope and DXM1200 camera) using Metavue software (Universal Imaging). Lateral wound boundaries were determined by the presence of intact hair follicles and organized epidermis and dermis compared with few or no hair follicles, altered epidermal/dermal organization, and disorganization of collagen fibers within the wound. Wound volume was estimated by interpolation from the wound areas measured in every 10th section (ie, every 70 μm) as described. The area of anti-CD31 immunolabeled blood vessels in the wound and wound size were measured digitally as above, and the vessel density (vesSEL area/wound area) was calculated. The number of vessels per wound area was also computed. Data were compared among groups using an unpaired t test (for 2 groups) or one-way ANOVA (for multiple groups) with a Bonferroni post hoc analysis as indicated and P<0.05 considered statistically significant.

Results
Wound Morphology
H&E-stained histological sections demonstrated marked morphological differences in uninjured skin of C57Bl/6 and Lepr^db mice, as previously described. The dermis of C57Bl/6 mice (Figure 1A) was thicker than that of Lepr^db mice (Figure 1D), whereas the reverse was true of the subcutaneous fat layer, which was extremely thick in Lepr^db mice. Histological differences between the 2 were also apparent in full-thickness 6-mm punch biopsy wounds. At 14 days, the epidermis in most Lepr^db wounds (Figure 1E) was much thicker than in C57Bl/6 wounds (Figure 1B), and collagen fibers appeared to be less dense and less evenly arranged in the diabetic than nondiabetic animals (data not shown). However, all of the wounds had closed and were smaller than the starting wounds.

mHPCs Improve Wound Healing
Three days after wounding, just before the start of wound revascularization, mHPCs or vehicle were injected under wounds. The mHPCs were 28.8% to 33.6% sca-1, as assessed by FACS. Wounds were harvested 11 days later (14 days after wounding). Treatment with mHPCs resulted in significant morphological changes in the Lepr^db wounds relative to vehicle controls. H&E (Figures 1E through 1F) and Masson’s trichrome-stained sections (Figure 2) demonstrate reduced epidermal and dermal thicknesses in mHPC-treated Lepr^db wounds. Masson’s trichrome-stained sections reveal denser, more organized collagen fibers, arranged parallel to the surface in HPC-treated Lepr^db wounds (Figure 2B) relative to controls (Figure 2A). No significant morphological...
changes were apparent in the mHPC-injected nondiabetic wounds relative to untreated controls (Figures 1B and 1C).

Treatment with mHPCs dramatically decreased wound size in Lepr db mice. Whereas wound volumes were 38% (±5.3%) of the original volume in untreated mice, they decreased to 20% (±2.1%) of original size in mHPC-treated mice (Figure 2C). That is, average wound size in treated mice was approximately half (51%; \( P < 0.01 \)) that of untreated controls. In contrast, no significant effect on wound size was observed in nondiabetic mice with mHPC treatment (11.2 ± 1.1%) versus controls (14.2 ± 1.3%) (Figure 2C).

**mHPC Injection Increases Wound Vascularization**

A series of histological sections of uninjured and wounded skin was immunolabeled with anti-CD31 antibodies to delineate blood vessels (Figures 1G through 1L). The vessels were traced digitally to determine vascular volume density (vascular volume/wound volume) in normal and injured skin. ANOVA comparison of diabetic versus nondiabetic wounds indicated that volume density significantly increased in diabetic skin (\( P < 0.05 \)), but post hoc analysis showed that this difference was attributable solely to increased vessel volume density in mHPC-treated Lepr db wounds relative to all other groups (\( P < 0.01 \)) (Figures 1G through 1L and 3A). Vascular volume density in the wounds of treated diabetic mice increased 1.7-fold relative to that in untreated mice (Figures 1K and 1L), which is the same fold increase previously observed for vascular volume density in CD34+ cell–treated wounds in streptozotocin-injected (type 1 diabetic) mice. There were no statistically significant differences in vascular volume density among other groups, although treatment of C57Bl/6 mice with mHPCs approached significance (\( P = 0.06 \)) relative to C57Bl/6 controls. (Figures 1H, 1I, and 3A).

To determine whether the increased vascular volume density resulted from increased vessel number or from vessel size, the vessel density (number of vessels per wound area) in the wounds was determined and compared by ANOVA among the groups (\( P < 0.01 \)). Post hoc analysis revealed that vessel density in mHPC-treated and untreated controls was similar in nondiabetic wounds (Figure 3B). In contrast, treatment of Lepr db mice with cells led to an increase in vessel density relative to nondiabetic mice (\( P < 0.01 \)), but although there was a trend toward increased vessel density between treated and untreated diabetic mice, it did not reach statistical significance (\( P = 0.06 \)) (Figure 3B). This suggests that increased vessel size is a major factor in the observed increase in vascular area density, but increased vessel number may also play a role. This is analogous to treatment of streptozotocin-injected mice with CD34+ cells, which induces increases in both vessel number and size at day 14.
Impaired Function of mHPCs Derived From Diabetic Mice

Because cells from nondiabetic mice both increased vascularization and improved wound healing in diabetic mice, we next investigated whether mHPCs from diabetic mice have the same potential. This was critical to understanding if and how type 2 diabetes impacts bone marrow cell function. As with cells derived from nondiabetic mice, wound size was reduced significantly in Lepr<sup>db</sup> mice injected with mHPCs from Lepr<sup>db</sup> mice compared with controls (Figure 4A). Surprisingly, however, a reduction of wound vascularity was readily apparent in histological sections (Figure 4B). The vessel density was significantly reduced in Lepr<sup>db</sup> mice treated with Lepr<sup>db</sup>-derived cells compared with the other 2 treatment groups (Figure 4C). In addition, vascular volume density was less than one third that of buffer control wounds and less than one sixth that of wounds treated with cells derived from nondiabetic mice (Figure 4D).

mHPC-Induced Inflammatory Response

One mechanism by which the mHPCs could alter wound healing and vascularization is through changes in the inflammatory response. Thus, we harvested wounds 5 days after wounding, that is, 2 days after injecting cells. H&E staining showed large scabs present in all wounds, and in both groups the inflammatory response was characterized by mononuclear cellular infiltration (mainly neutrophils), macrophage migration, and collagen matrix deposition. No differences were apparent in the inflammatory response in mHPC-treated wounds versus control wounds except that slightly fewer macrophages seemed to be present in mHPC-treated wounds. To examine this more closely, sections were immunostained with ER-MP23 antibody to detect monocyte/macrophages in day 5 wounds (ie, 2 days after mHPC injection). A small difference in the nature of the inflammatory infiltrate was observed. Although the number of immunolabeled cells in both buffer and mHPC-treated wounds was similar, the labeled cells in the former (Figure 5A) tended to be much larger than those in the latter (Figure 5B). Moreover, whereas the cells were dispersed throughout buffer-treated wounds, they tended to cluster, especially near blood vessels entering the wound bed, in mHPC-injected wounds (Figure 5).
Lack of Incorporation of mHPCs Into Wound Tissue

Although we and others have reported that HPCs integrate into the vasculature, reports vary widely as to the extent of this incorporation, such that integration seems to be dependent on the model. To investigate possible incorporation of mHPCs into the vasculature in these wounds, cells derived from nondiabetic mice expressing the EGFP protein in all cells were injected under wounds of non-EGFP C57Bl/6 and Leprdb mice as above. Skin was harvested 14 days after wounding and immunolabeled with anti-GFP to detect bone marrow–derived cells and Bandeira Simplicifolia lectin B 4 to label the mouse vasculature. Although bone marrow–derived cells were present in the wound vasculature, they were rare, occurring at a frequency of \(0.1\%\) (data not shown). Additionally, because monocytes may bind this lectin, we cannot be sure that the rare cells that seemed to be integrated into the vessels that were colabeled with anti-GFP and lectin were actually endothelial cells.

Discussion

Our data suggest that some but not all functions of adult stem/progenitor cells are impaired by type 2 diabetes. The cells retain their ability to promote wound healing in small wounds, but not only are they incapable of promoting vascularization, they actually inhibit it. Significantly, these surprising findings show that neovascularization and wound healing are not always coupled. Additionally, there is little evidence of stem/progenitor incorporation into wound structures, suggesting that improved healing is mediated through mechanisms other than direct differentiation and integration of the cells.

Impaired neovascularization is a clinically significant problem in diabetic patients, and therapies designed to improve vascularization can improve outcomes in patients with diabetic ulcers and poorly healing wounds. We showed previously that injection of circulating nondiabetic human CD34\(^+\)-enriched cells into type 1 diabetic nude mice accelerated restoration of blood flow to ischemic limbs of diabetic but not nondiabetic mice.7 In a more recent study in a model of type 1 diabetes, we found that the same cells promote vascularization and skin wound healing. 13

In this study, we examined the effects of the related mHPCs on both vascularization and healing of skin wounds in Lepr\(^{db}\) mice, a model for type 2 diabetes. Although angiogenesis is not impaired in Lepr\(^{db}\) mice, we anticipated that increased vascularization would be a harbinger of improved wound healing, because this is typically the case. Consistent with this and with the aforementioned studies, our data demonstrate that mHPCs from nondiabetic C57Bl/6 mice greatly increase vascularization in Lepr\(^{db}\) mice but not in C57Bl/6 mice, at least at the time point examined. Improved vascularization correlates with improved wound healing as assessed by both collagen deposition and wound volume in the mHPC Lepr\(^{db}\) mice. This improvement is likely to be physiologically significant, because treatment reduces wound size by almost half compared with controls.

Despite this correlation between healing and vascularity in mice treated with nondiabetic mHPCs, our data show that these 2 phenomena are not necessarily linked. A profound inhibition of vascular growth by Lepr\(^{db}\)-derived mHPCs in Lepr\(^{db}\) mice not only did not inhibit wound healing, but wound healing in mice treated with these cells improved as much as in Lepr\(^{db}\) mice treated with C57Bl/6-derived cells. That is, there is no coupling between vascularity and wound healing in these mice, suggest-
ing that previously observed correlations between revascularization and wound healing may have little to do with improved tissue perfusion. Why the two are so consistently linked becomes an intriguing question.

The data point out 2 other interesting questions. What properties of the injected mHPCs promote wound healing, and how does diabetes alter mHPCs such they no longer induce vascularization but rather inhibit it? Of course it is possible that the observed mHPC dysfunction is attributable to an inability of the cells to respond to leptin rather than the hyperglycemic state of the mice, and the data will need to be confirmed in another model. However, earlier data demonstrating impaired function of EC progenitors from type 1 diabetic patients support a role for diabetes in the mHPC dysfunction.7

**Figure 4.** Effects of Lepr<sup>db</sup>-derived mHPCs on wounds. Wounds in skin of Lepr<sup>db</sup> mice 14 days after wounding treated with buffer or HPCs from C57Bl/6 (HPC) or Lepr<sup>db</sup> (Diab HPC) mice 3 days after wounding were examined. A, Morphometric analysis of wound volume. B, Brightfield micrograph of histological section of Lepr<sup>db</sup> wound treated with mHPCs from an Lepr<sup>db</sup> mouse. Sections of 7 µm labeled with anti-CD31 antibodies visualized with Vector Red (red) and stained with hematoxylin. Bar=50 µm. C, Morphometric analysis of the number of blood vessels per unit area of treated injured skin. D, Morphometric analysis of volume density of blood vessels in treated injured skin. Error bars = SEM. *P<0.01.

mHPCs could affect wound healing through changes in the inflammatory response, but we observed no obvious morphological differences in the inflammatory responses, and the overall number of neutrophils and inflammatory cells present seemed to be similar in buffer- and mHPC-treated wounds. However, immunostaining with the monocyte/macrophage antigen revealed one intriguing difference. Whereas in the buffer-treated wounds, large immunolabeled cells were distributed fairly evenly throughout the wound, in the mHPC-treated wounds, similar numbers of smaller labeled cells were present but clustered. One interpretation is that monocytes in the buffer-treated wound migrated and differentiated into macrophages, whereas in the mHPC-treated wounds, mono-

**Figure 5.** Monocyte/macrophages in wounds. Fluorescence micrographs of 7-µm sections of wounds in skin of Lepr<sup>db</sup> mice 14 days after wounding and treated 3 days later with buffer (A) or C57Bl/6-derived mHPCs (B). Fluorescence (white) indicates cells labeled with anti-ER-MP23 antibodies to detect monocyte/macrophages.
cyte migration and differentiation were inhibited. Exactly how this might affect healing remains to be examined.

It is interesting that the observed increased vascularity in nondiabetic mHPC-treated wounds is not principally attributable to an increase in vessel number but instead vessel size. We saw a similar effect of human CD34+ cells on vessel size in skin wounds in nude mice. Moreover, injection of CD34+ cells into the ischemic limb of diabetic mice led to significant increases in blood flow within 2 days, indicative of the presence of resistance vessels.

That is, bone marrow–derived cells may play an important role in increasing blood flow and only a lesser or indirect role in improving tissue perfusion. How the cells accomplish this, e.g., via opening of cryptic channels, inducing vasodilation, enlargement of existing vessels, or formation of large vessels, is unclear.

That diabetic bone marrow cells can inhibit neovascularization could have clinical significance. Autologous bone marrow treatment may provide functional improvement in some settings, but such treatment could actually exacerbate circulatory problems in at least a subset of diabetic patients. Furthermore, whereas wound healing and vascularization may be decoupled in small wounds, it seems less likely that this will be strictly true in larger wounds. Thus, it might be necessary to allograft stem/progenitor cells from nondiabetic donors into type 2 diabetic patients to achieve successful therapeutic outcomes.

Acknowledgments
This study was made possible by Juvenile Diabetes Research Foundation grant 1-2001-534, NIH grant DK59223, and a pilot grant from NIH DK25295 Diabetes and Endocrinology Research Center. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

References
Lepr<sup>db</sup> Diabetic Mouse Bone Marrow Cells Inhibit Skin Wound Vascularization but Promote Wound Healing

Vesna Stepanovic, Ola Awad, Chunhua Jiao, Martine Dunnwald and Gina C. Schatteman

Circ Res. published online May 1, 2003; Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2003/05/01/01.RES.0000074906.98021.55.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/