Diabetes mellitus is associated with micro- and macrovascular dysfunction and disease (20), including impaired cardiac vascular angiogenesis that may contribute to severe clinical cardiovascular disease (1, 9, 30). Mechanistically, diabetes has been linked to altered nitric oxide signaling (5, 27), oxidative stress (17, 31), and the formation of advanced glycation end products (3, 4, 18), as well as dyslipidemia (2, 13). However, despite the identification of such disease-related mechanisms, the specific cell targets mediating the impairment in vascular function remain to be defined.

Diabetes has been shown to impact the function of both endothelial cells as well as endothelial progenitor cells (EPCs). Clinical investigations have revealed that diabetes is associated with impaired endothelial cell migration in vitro (10) as well as impaired endothelium-dependent vasodilation in vivo (12). Diabetes has additionally been linked with decreased numbers of circulating EPCs as well as impaired angiogenic function of diabetic-derived EPCs (15). Moreover, compared with cells from healthy donors, EPCs isolated from diabetic patients exhibit impaired ability to bind to activated endothelial cells in culture and promote blood flow to vascular structures in ischemic limbs (22, 23). Such changes in endothelial-EPC interactions may be critical in the mechanisms underlying the impairment in cardiac vascular function and may contribute to the inverse correlation of EPC levels and cardiovascular disease risk (24). Indeed, the local administration of bone marrow-derived CD34-positive (CD34+) cells from control animals improves blood flow and wound healing in hindlimb ischemia and skin wound models in diabetic animals (16, 19), suggesting that a primary defect in EPCs may similarly contribute to impaired cardiac angiogenic function associated with diabetes.

The present investigations sought to define the vascular and endothelial compartment(s) underlying the diabetic dysregulation in vascular function, employing a cardiac allograft model which allows for the assessment of the specific contribution of local vascular and EPC-mediated cardiac angiogenic activity in the diabetic milieu while maintaining a wild-type myocardial environment. Previously, this research approach has been utilized to elucidate the role of impaired expression of platelet-derived growth factor (PDGF) by endothelial cells as well as EPCs in age-associated depression in cardiac angiogenic function (7, 8). Specifically, we hypothesized that studies in the pinnal allograft model would identify the vascular/endothelial compartment(s) that underlie the diabetic impairment in cardiac vascular function. Indeed, because this model has facilitated the advancement of novel cardioprotective approaches (7, 29), the results of pinnal allograft studies in diabetic mice could direct the development of molecular- and/or cellular-based approaches that may reduce diabetic-related cardiovascular diseases.
MATERIALS AND METHODS

Animals. All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University, which follows federal and state guidelines.

Diabetic induction. To generate diabetic mice, 3-mo-old male C57Bl/6 and B6.129Sv-Gtrosa26 (Rosa) mice were randomized to receive injections intraperitoneally with 40 mg/kg of streptozotocin (STZ) dissolved in 0.1 M sodium citrate buffer, pH 4.5, or vehicle only in the case of control animals, for 5 days as previously described (14). Three days after the final injection, the animal’s blood glucose was measured by using an Accu-Chek II Advantage Glucometer (Roche Diagnostics). Diabetic animals with a serum level of glucose <225 mg/dl received an additional 2 days of STZ at the same dose.

Cardiac allograft angiogenesis assay. To test the effects of diabetes on cardiac angiogenic function and its potential modulation of vascular function in different host mice, we employed a cardiac allograft model. This model allows for the assessment of disease-associated alterations in cardiac angiogenesis while controlling for the functional and structural integrity of the (wild-type neonatal) heart being vascularized (6–8). Briefly, 8 wk after STZ or control treatment, the mice were transplanted by a blinded investigator with cardiac allografts explanted from neonatal (1 day old) C57Bl/6 pups, which were inserted into a subcutaneous pocket that had been surgically created in the host pinnae as previously described (6–8). On posttransplant days 3 and 7, the allograft transplants were tested for viability and function as assessed by a blinded investigator scoring visual integrity and electrocardiographic (ECG) activity in the transplanted pinnae as previously described (6). A transplant was considered functional only if it was both visually intact and demonstrated sustained chronotropic activity of ≥1 Hz. Blood flow to the cardiac allografts in the control and diabetic hosts was also quantified on posttransplant days 3 and 7 by a blinded investigator employing laser-Doppler measurements with a single-channel Advance Laser Flowmeter ALF21/21D (Advance, Tokyo, Japan) equipped with a contact C-Probe (range: 0–100 ml·min^{-1}·100 g tissue^{-1}) as previously described (6). On the basis of the average mass of the neonatal cardiac tissue transplanted into the pinnae (2 mg), the flow into the allografts was calculated from the laser-Doppler measurements (in ml·min^{-1}·100 g tissue^{-1}). Additional sets of both diabetic and control animals received injections of 100 ng/10 μl of PDGF-AB into the pinnae 24 h before allograft transplantation to assess the potential reversibility of vascular defects in the diabetic animal. The sample size was n ≥ 7 per study condition.

Bone marrow-mediated cardiac angiogenic activity. To investigate diabetes-associated defects in the ability of bone marrow-derived cells to promote cardiac angiogenic activity, transplantation of bone marrow cells derived from young diabetic donor animals into angiogenically deficient senescent (18 mo old) hosts was performed. Briefly, bone marrow cells isolated from diabetic or control C57Bl/6 as well as diabetic or control Rosa mice were injected (1 × 10^6 cells/injection) into the tail veins of 18-mo-old intact, unirradiated, C57Bl/6 female host mice as previously described (8). Mice receiving PBS injections served as controls. Cardiac angiogenesis was tested 7 days after bone marrow transplantation by means of the cardiac allograft assay. The sample size was n ≥ 7 per study condition.

To further test the potential of bone marrow-derived cells to reverse diabetes-associated vascular dysfunction, sets of diabetic animals were injected with bone marrow cells isolated from young male control Rosa mice. Groups of control and diabetic mice pretreated with PDGF received bone marrow-derived cells (1 × 10^6 cells/injection) followed by cardiac allograft transplantation 7 days later as described above (sample size was n ≥ 10 per study condition).

RESULTS

Impaired cardiac allograft function in diabetic hosts. To investigate the potential impact of diabetes on cardiac angiogenic function without affecting endogenous cardiac function and related physiology, our studies employed a cardiac allograft transplantation model. In this assay, syngeneic neonatal hearts are engrafted subcutaneously into the pinnae of host mice. Through host-dependent angiogenesis, the cardiac tissue is vascularized, demonstrating independent chronotropic activity as a physiological measure of allograft function and viability (Fig. 1A). When compared with transplants in control hosts, the induction of chronotropic activity in the diabetic engrafted hearts was significantly delayed (Fig. 1B), which correlated with significantly lower blood flow to the cardiac tissue 3 days after transplantation. Notably, by 7 days, the chronotropic activity and blood flow to the transplants in the diabetic hosts were similar to measurements in control mice. On the basis of the importance of tissue vascularization for electrical activity of the cardiac allografts (6–8), these findings suggested that diabetes may impair cardiac angiogenic function. Moreover, on the basis of the capacity of this model to allow for the isolation of specific components of angiogenesis, the cardiac allograft assay could facilitate the evaluation of the mechanisms and potential reversal of the functional alterations in the cardiac angiogenic function of the diabetic host.

Diabetic impairment in bone marrow-mediated cardiac angiogenesis. To identify the potential cellular basis of the impairment in cardiac angiogenic function in the diabetic mice, our studies attempted to isolate the impact of diabetes on bone marrow-derived, cell-mediated function in a senescent transplant model. To this end, we have previously demonstrated that EPCs derived from transplanted young control bone marrow cells home to the bone marrow of intact, unirradiated syngeneic 18-mo-old mice and give rise to EPCs that can restore senescent cardiac angiogenic function (8). In this model, cells from diabetic and control mice revealed a similar capacity to home to the bone marrow of the aging mice (Fig. 2). However, cells derived from the wild-type bone marrow demonstrated a significantly greater capacity to home to the pinnaal cardiac allografts and promoted the chronotropic function of the transplanted cardiac tissue, suggesting that alterations in bone marrow-derived cells, including EPCs, may contribute to the diabetic impairment in cardiac angiogenic function.

Diabetic alterations in peripheral vascular function. Our studies then focused on the changes in local vascular function...
that could contribute to the diabetic impairment in cardiac angiogenic function. On the basis of previous studies demonstrating the capacity of local pinnal treatment with PDGF-AB to improve both cardiac blood flow (6) and cardiac function (7), the diabetic mice were pretreated with PDGF-AB 1 day before cardiac transplantation. PDGF-AB enhanced the function of the cardiac transplants in the control mice, similar to as we previously reported (6, 7) (Fig. 3). Importantly, however, PDGF-AB not only did not improve the function of the cardiac allografts in the diabetic hosts but resulted in a significant decrease in the function of the transplants (Fig. 3).

Bone marrow cell-PDGF synergism in restoration of cardiac allograft function. In an attempt to reverse the impairment of cardiac allograft function imparted by alterations in the diabetic vasculature, including bone marrow-derived cells, the diabetic mice were transplanted with bone marrow cells isolated from healthy control animals. Transplantation of these cells did not reverse the diabetic delay in allograft function (Fig. 3). The combination of control bone marrow cell transplantation and pinnal PDGF-AB pretreatment did, however, reverse the PDGF-AB-induced impairment in allograft activity.

To probe the potential mechanisms mediating the synergistic restoration of cardiac angiogenic function, the tissue distribution of transplanted cells with and without PDGF-AB pretreatment of the diabetic tissue was examined. This histological analysis demonstrated significantly greater numbers of wild-type, donor-derived cells populating the diabetic bone marrow compared with transplants into control mice (Fig. 4). Treatment of both control and diabetic mice with PDGF-AB resulted in increased donor-derived cells in the host bone marrow. Moreover, the pinnal pretreatment with PDGF-AB resulted in an alteration in the bone marrow patterning of the donor cells with a sinusoid distribution of transplanted donor cells in the host marrow that was not observed in the control mice or the diabetic host receiving bone marrow alone. There was no significant homing of the transplanted bone marrow cells to the cardiac allografts in either the intact control or diabetic mice. Similarly, control mice treated with PDGF-AB and labeled bone marrow cells did not reveal significant recruitment into the transplanted cardiac allografts. In the mice receiving both wild-type bone marrow and pinnal PDGF-AB pretreatment, histology confirmed the cardiac recruitment of the transplanted bone marrow-derived EPCs, which costained for vWF.

DISCUSSION

The present in vivo studies reveal the important combined role of bone marrow-derived cell dysregulation and altered vascular function in the impairment in cardiac angiogenic function observed in diabetic mice. Specifically, these studies demonstrate a primary defect in the ability of cells derived from diabetic bone marrow cells to promote cardiac vascularization with resultant decreases in allogenic cardiac tissue activity. Moreover, the diabetic milieu impairs the in vivo angiogenic function of PDGF-AB pathways and similarly suppresses the cardiac recruitment of bone marrow-derived cells, including EPCs, derived from transplanted control bone marrow. Importantly, the combination of control bone marrow with pinnal PDGF-AB-pretreatment results in improvement in the cardiac angiogenic potential of the transplanted bone marrow cells and promotes the survival of transplanted cardiac tissue.

The STZ treatment mice provided a quantitative approach to study the impairment as well as potential improvement in cardiac angiogenic function in diabetic mice. To this end, the failure of the diabetic bone marrow cells to restore vascularization of the cardiac allografts in the older mice may parallel the dysfunction of cells derived from the aging bone marrow. Indeed, diabetes has been suggested to be a model of vascular aging (25, 26), with accumulated effects of changes such as oxidative stress, altered signaling pathways, and advanced glycation end products that may contribute to the impairment in both local endothelial cell- and EPC-mediated angiogenic function (7, 8). However, the failure of the control bone marrow to fully restore cardiac vascular function suggests that the diabetic changes may be more extensive than those observed with physiological aging.

The deleterious effects of PDGF-AB treatment on cardiac allograft function in the diabetic hosts demonstrate the impor-
tance of local shifts in diabetic vascular function that differ significantly from age-related physiological changes. In the nondiabetic state, PDGF-AB enhances angiogenesis and promotes the function of the cardiac allografts in young (6) as well as older mice (7, 29). PDGF-AB pretreatment of the diabetic pinnal tissue, however, resulted in an impairment in cardiac allograft function, suggesting that the signaling pathways and/or downstream proangiogenic cascades induced by PDGF-AB are dysfunctional in the local diabetic vascular milieu. To this end, previous studies (7, 8, 29) have established the interrelationship between PDGF-AB- and EPC-mediated angiogenic function involving the induction of PDGF receptors to promote cardiac angiogenesis and cardioprotection in rodent model systems. Moreover, in addition to potential changes involving peripheral endothelial cells and circulating EPCs, the actions of PDGF on other vascular cell targets, including pericytes and smooth muscle cells (11), may result in the compromise of the transplanted cardiac tissue.

The beneficial synergism of wild-type bone marrow and PDGF-AB demonstrates the importance of local and systemic

**Fig. 2.** Assessment of ability of diabetic-derived donor cells to promote cardiac angiogenesis. A: representative photographs of pinnae of 18-mo-old angiogenically deficient senescent mice administered no bone marrow (No BM) or bone marrow from control (CTL BM) or diabetic donors (DM BM) before cardiac allograft transplantation with representative ECGs below (5-s intervals shown). Of note, allografts without pretransplantation of young bone marrow cells resulted in loss of cardiac transplants and surrounding pinnal tissue. Transplants with young control bone marrow were intact with a high percentage of ECG (functional) activity. Transplants in mice receiving bone marrow from diabetic mice were intact but lacked robust ECG function. B: quantification of percentage of allograft function 7 days after allograft transplantation in animals receiving No BM, CTL BM, and DM BM. *P < 0.05 vs. No BM; n ≥ 7 mice per group. C: representative micrographs 7 days after cardiac allograft transplantation from 18-mo-old host mice administered Rosa control or diabetic bone marrow-derived cells. Eosin-stained slides demonstrate incorporation of transplanted cells into host femur and peripheral pinnae. D: quantification of density of β-galactosidase (β-Gal)-positive (+) donor cells present in host femur and pinnae scored per ×40 high-power field (HPF). *P < 0.05 vs. CTL BM.

**Fig. 3.** Assessment of allograft ECG activity in control and diabetic mice treated by transplantation of control bone marrow (BMT) (without prior myeloablation) and/or pinnal injection of PDGF-AB before cardiac allograft transplantation. Total percentage of transplants with independent, normal ECG activity was scored at 3 (3d) and 7 (7d) days after cardiac allograft transplantation. *P < 0.05 vs. diabetic and control alone (7d); **P < 0.05 vs. control alone (3d); n ≥ 10 mice per group.
pathways in diabetic vascular impairment. Our studies utilizing bone marrow-derived cells from nondiabetic mice reveal that bone marrow-derived cells, such as vWF+ EPCs, are a target of local PDGF-AB pathways in cardiac angiogenesis, with the impairment in the diabetic EPCs potentially underlying the loss of PDGF-AB-mediated cardiac angiogenic function. Indeed, the capacity of wild-type bone marrow to restore the cardioprotective effects of PDGF-AB in the diabetic pinnal allografts demonstrates the importance of bone marrow-peripheral vascular interactions in the regulation of local vascular function. Mechanistically, such regulation may parallel the autocrine and paracrine pathways of EPCs that can govern PDGF-AB induction in senescent cardiac angiogenesis (6, 7, 29) and stem cell differentiation in bone marrow-mediated cardiogenesis (28). Moreover, the induced sinusoidal distribution of the donor cells in the diabetic bone marrow after pinnal PDGF-AB pretreatment suggests that the interaction between the wild-type bone marrow-derived cells, including EPCs, and PDGF-AB in the pinnal vasculature may induce positive feedback loops to the chimeric bone marrow induced by local angiogenic pathways. Indeed, the increased density of donor-derived cells in the bone marrow of control mice treated with PDGF-AB demonstrates the importance of peripheral growth factor pathways in bone marrow function. To this end, previous studies have demonstrated that PDGF-AB can expand populations of CD34+ progenitor cells in vitro (21), which have been shown to promote vascularization of ischemic hindlimbs after injection into diabetic models (16). These findings suggest that PDGF-AB may alter the function of the EPCs after recruitment to the angiogenic foci, potentially inducing systemic growth factors such as stromal cell-derived factor 1 or vascular endothelial growth factor and/or the potential rehoming of the peripherally stimulated EPCs to the bone marrow to promote the recruitment of additional cells to synergize with the local vasculature to enhance cardiac angiogenesis in the diabetic milieu.

The synergism between systemic, bone marrow cell-mediated function and PDGF-AB pathways may provide significant cardioprotection for the diabetic heart. The local and systemic actions of PDGF-AB to enhance EPC migration in the microvasculature as well as the bone marrow, respectively, suggest this synergism will improve function in other vascular beds, including the microvascular circulation, to potentially reverse the diabetic impairment in wound healing. Further studies to more fully define the mechanisms mediating the interactions between bone marrow-derived and local vascular cells, in the peripheral vasculature as well as the endogenous bone marrow, may facilitate the development of novel approaches to decrease the impact of diabetes on cardiac and vascular diseases.

Fig. 4. Assessment of relative tissue density of transplanted donor cells derived from CTL BM in a diabetic host with or without PDGF-AB pretreatment. A: representative micrographs 7 days after cardiac allograft transplantation in host mice administered Rosa control bone marrow-derived cells. X-gal stains of host femur and pinnal cardiac allografts in CTL or DM hosts, with and without coadministration of pinnal PDGF-AB pretreatment. Arrows show donor cells within transplanted cardiac allografts in animals receiving PDGF-AB 24 h before transplantation. B: quantification of number of transplanted β-Gal(+) Rosa-derived control cells in host femur, pinnae, and cardiac allograft scored per ×40 HPF. *P < 0.05 vs. control; **P < 0.05 vs. CTL, DM, and CTL + PDGF-AB. C: representative panels of von Willebrand factor immunostaining (diaminobenzidine) and donor-derived cells (X-gal) costaining in a perivascular distribution (arrows, en face microvascular staining) of pinnal cardiac allografts in diabetic mice.
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