Role of the renin angiotensin system on bone marrow-derived stem cell function and its impact on skeletal muscle angiogenesis

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Submitted 17 February 2010; accepted in final form 25 May 2010

The formation of new blood vessels is essential for a variety of physiological processes as well as for tissue repair and remodeling during acute and chronic ischemic vascular diseases. Rapid revascularization of injured, ischemic, and regenerating organs is essential to restore organ function. Despite great advances in both medical and surgical management of patients with ischemic-related diseases, failure in the revascularization process is still considered a major issue and is frequently associated with a poor prognosis. Over the past decade, intensive efforts have been undertaken to better understand the underlying mechanisms regulating blood vessel growth and to develop new therapeutic options to promote revascularization of ischemic tissues.

Recently, new strategies using transplantation of autologous bone marrow cells (BMC) have been shown as an innovative and promising therapeutic approach to induce neovascularization in ischemic tissues in adults (19, 29, 33, 50, 51). Several studies have shown that endothelial progenitor cells (EPC), identified in the bone marrow, augment reparative neovascularization either through differentiation into mature endothelial cells (EC) or indirectly through paracrine stimulation of resident EC proliferation (43, 60). Numerous experimental studies have shown that the transplantation, as well as the therapeutic mobilization of the EPC to the sites of injury, restored tissue vascularization after ischemic events in limbs, retina, brain, and myocardium (17, 18, 28, 40, 53–55). These studies have created tremendous enthusiasm in the stem cell research field. However, many of the benefits found with the EPC therapy in experimental animals were not replicated in clinical trials (30, 37). This seems to be due to the fact that the primary studies showing the role of the EPC in neovascularization were performed in healthy animals with experimentally induced vascular injury, whereas vascular events often occur in patients with cardiovascular disease risk factors and endothelial dysfunction.

Recent studies have shown reduced EPC availability and altered EPC function and differentiation in patients at increased cardiovascular disease risk may negatively influence current therapeutic strategies involving autologous stem cell transplantation (22, 58). EPC dysfunction has now been shown in a large number of diseases including diabetes, atherosclerosis, stroke, and hypertension (36, 42, 56, 58). The mechanisms underlying the EPC dysfunction are not clear. However, the impairment in EPC proliferation, adhesion, and angiogenic properties may underlie new mechanisms involved in disease pathogenesis or vascular complications. Development of new strategies to restore EPC function and consequently increase EPC engraftment and/or mobilization may substantially impact angiogenic stem cell-based therapy.

The present study takes advantage of the immune-compatible chromosome substitution rats to provide new insight into the mechanisms underlying reduced EPC availability and/or altered EPC function and differentiation. The Dahl salt-sensitive rat (SS/Mcwi) is an animal model for salt-sensitive hypertension and rarefaction. We have previously shown that the skeletal muscle angiogenesis induced by electrical stimulation is significantly impaired in the SS/Mcwi rats regardless of the salt intake (3, 9) and that this angiogenesis is restored in the consomic SS-13BN/Mcwi rat by a renin dependent mechanism (3). Although the dysregulation of the renin angiotensin system (RAS) has been implied in the poor angiogenesis response as well as vascular dysfunction in the SS/Mcwi rats (3, 9, 12, 13, 35, 38), little is known about the role of the RAS on bone marrow-derived endothelial cell (BM-EC) function and stem cell-based therapy. In the present study we hypothesized that low RAS activity present in SS/Mcwi rats will impair BM-EC function and consequently the angiogenic efficacy of bone marrow stem cell therapy. Understanding how the RAS affects the EPC function may help to identify optimal stem
cell therapy to treat the reduction in vessel density that occurs in several forms of low renin hypertension in humans (20, 32, 47).

MATERIAL AND METHODS

Bone marrow cell isolation. The Medical College of Wisconsin (MCW) Institutional Animal Care and Use Committee approved all animal protocols. The SS/Mcwi rat strain is an inbred substrain derived from the Dahl-S rat. The SS-13<sup>BN</sup>/Mcwi consomic rat strain was derived from BN/Mcwi rats and SS/Mcwi rats by replacement of SS chromosome 13 with BN chromosome 13 on the SS genetic background; the origin of both of these strains has been described previously (8). Animals were housed and cared for in the MCW Animal Resource Center and were given low salt diet (0.4% NaCl) and water ad libitum. Donor rats were killed with an overdose of pentobarbital, both femurs and tibias were surgically dissected, and the adhering tissues were completely removed. Both ends of the bones were excised, and bone marrow cells were harvested by flushing with RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 20% fetal calf serum, using a 23-gauge needle. The BMC were gently resuspended with an 18-gauge needle and filtered through sterile 100 μm nylon mesh. Red blood cells were lysed with red blood cell lysis buffer (BioLegend) for 5 min at room temperature. Cells were washed in PBS three times and then resuspended at 6 × 10<sup>6</sup> cells in 100 μl of PBS for transplantation or Western blot analysis.

Electrical stimulation surgery and BMC implantation. The tibialis anterior (TA) and extensor digitorum longus (EDL) muscles of the recipient and sham-injected animals were electrically stimulated for 8 h per day for 7 consecutive days as previously described (34). Briefly, SS/Mcwi rats were anesthetized with intramuscular injection of a mixture of ketamine (100 mg/kg), xylazine (50 mg/kg), and acepromazine (2 mg/kg). BMC (6 × 10<sup>6</sup> cells) or PBS was injected into three to four different areas of the right TA muscle of the SS/Mcwi rats. The recipient rats were randomly divided into five groups. 1) Control, SS/Mcwi rats that received 0.1 ml of PBS injection; 2) BMC-SS, SS/Mcwi rats that received BMC from SS/Mcwi rats; 3) BMC-SS<sup>13BN</sup>, SS/Mcwi rats that received BMC from the SS-13<sup>BN</sup>/Mcwi; 4) BMC-SS ANG II, SS/Mcwi rats that received BMC from the SS/Mcwi rats infused with a subpressor dose of ANG II; 5) BMC-SS saline, SS/Mcwi rats that received BMC from the SS/Mcwi rats infused with saline. Immediately after the cell or PBS injection a miniature battery-powered stimulator was implanted subcutaneously in the thoracolumbar region of the rats and a pair of electrodes was tunneled under the skin from the stimulator to the right lower hindlimb muscles to promote muscle contractions. On the following day the electrical stimulators were turned on. Animals were euthanized 7 days following the onset of stimulation for analysis of angiogenesis. A schematic illustration of the bone marrow injection protocol and vessel density analysis is given in Fig. 1A. A total of 61 animals were used in this study. Numbers of animals for each group varied according to the experiment design and are indicated in the figure legends.

Morphological analysis of vessel density. The TA muscle from the unstimulated and stimulated hindlimbs were harvested and stained with rhodamine-labeled <i>G. simplicifolia</i> I lectin (Sigma) as previously described (11, 46). Briefly, the TA muscles from the unstimulated and stimulated hindlimbs were harvested and fixed in 0.25% formalin for 24 h. Each muscle was longitudinally sliced on a sliding microtome to a thickness of ~75 μM and stained in a 30 μg/ml rhodamine-labeled <i>G. simplicifolia</i> I lectin (Sigma) solution. After several rinses slices were mounted on microscope slides in water-soluble mounting media. Twenty images per muscle were taken at x200 under fluorescent light (excitation 555 nm and emission wavelength 580 nm). Image files were analyzed by Metamorph software using an overlaid 10 × 13 grid, and...
vessel density is expressed as the mean number of vessel-grid intersections per microscopic field (0.077 mm²).

**Monitoring of the implanted BMC in the ischemic hindlimbs.**

To show the incorporation of the cells into the skeletal muscle vasculature and their differentiation into EC, BMC (2 × 10⁷ cells/ml) were isolated from SS-13BN/Mcwi rat and labeled with of PKH-67 dye (Sigma) according to manufacturer’s instructions. In brief, samples were incubated at room temperature with 4 μM PKH-67 dye for 5 min with gentle mixing. Staining was terminated by addition of equal volume of RPMI media containing 20% FBS; cells were collected by centrifugation (400 g, 10 min, 4°C) and washed three times with PBS. The cell viability was tested by the trypan blue exclusion test. Cells were resuspended in 0.1 ml of PBS and injected into three or four different areas of the TA muscle of SS/Mcwi rats. After 7 days of electrical stimulation the TA muscle of the SS/Mcwi rats was collected and processed for lectin or CD31 staining as previously described (11, 46).

**In vivo ANG II infusion.** SS/Mcwi rats received ANG II at a subpressor dose of 3 ng·kg⁻¹·min⁻¹ or saline intravenously for 7 days as described previously (11, 21, 38, 41). Briefly, SS/Mcwi rats were anesthetized with intramuscular injection of a mixture of ketamine (100 mg/kg), xylazine (50 mg/kg), and acepromazine (2 mg/kg), a catheter was securely inserted into the left jugular vein, and the animals were allowed to recover for 24 h. Conscious rats were then connected to a multisyringe pump to provide a continuous intravascular infusion of saline alone or saline containing ANG II (3 ng·kg⁻¹·min⁻¹) for 7 days in their home cages. After 7 days the BMC were harvested and resuspended in PBS for implantation.

**Vascular endothelial growth factor Western blotting.** Whole BMC were harvested and then washed three times in cold PBS and lysed in RIPA buffer (150 mM NaCl, 1% NP 40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris base, pH 8.0) containing protease inhibitors (10 mmol/l sodium pyrophosphate, 100 mmol/l NaF, 1 mmol/l Na3VO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 mmol/l PMSF). Protein was separated in a denaturing SDS/4–20% polyacrylamide gel (20 μg per lane) and then blotted onto a nitrocellulose membrane. Membranes were incubated with a rabbit polyclonal antibody for vascular endothelial growth factor [VEGF (A-20), dilution 1:500; Santa Cruz, CA] for 4 h at room temperature and after serial washes (5 × 3 min in TBS-T), with the secondary antibody (anti-rabbit IgG, 1:3,000) for 1 h at room temperature. Immunoblots were visualized by chemiluminescence (Pierce Rockford, IL), followed by autoradiography. VEGF (A-20) is recommended for detection of the 189, 165, and 121 amino acid splice variants of VEGF. The 42 kDa band (VEGF dimer) was quantified. Membranes were stained with Ponceau S and expressed as relative densitometric values using the Image Quant software.

**BM-EC isolation, characterization, and tube formation assay.** Mononuclear cells (MNC) were isolated by gradient density centrifugation (Histopaque 1083, Sigma) according to the manufacturer’s protocol. After purification with three washing steps, 10⁷ MNC were plated on fibronectin-coated six-well plates. Cells were cultured in endothelial cell basal medium-2 (Clonetics) supplemented with EGM-2 single aliquots consisting of 10% FBS, VEGF, fibroblast growth fac-

tor-2, epidermal growth factor, insulin-like growth factor-1, ascorbic acid, and antibiotics. Adherent cells were washed on day 4 to remove unattached cells, and fresh growth medium was added. After 7 days in culture BM-EC were identified by typical EC morphology. However, it must also be acknowledged that the cell population used in the present study is heterogeneous but considered enriched in endothelial progenitor cells, which express EC markers including CD31 expression. BS-1 lectin, and incorporation of acetylated low-density lipoprotein with marked angiogenic properties. BM-EC were further imaged to confirm incorporation of acetylated low-density lipoprotein (Dil-ac-LDL, Biomedical Technologies) and binding of BS-1 lectin (FITC-labeled lectin from Bandeiraea simplicifolia, Sigma) as previously described (63). Immunofluorescence for detection of CD31 was performed using an anti-rat CD31 antibody (PharMingen) by a standard protocol as given by the manufacturer.

To investigate the ability of BM-EC to integrate into vascular structures, BM-EC (10⁴ cells) from SS/Mcwi rats infused with saline or ANG II were cocultured with human umbilical vein endothelial cells (HUVEC, 10⁴ cells) on Matrigel (BD Biosciences) as previously described (14). BM-EC were stained with Dil-ac-LDL (Molecular Probes, 5 μg/ml) for 1 h and then coplanted with HUVEC labeled with DAPI (20 μg/ml, Sigma) on Matrigel-coated 96-well plates. After 18 h the network formation was assessed using an inverted phase contrast microscope (Nikon, Tokyo, Japan). The total length of tubes was quantified in 2–5 random low-power fields per group. The incorporation of BM-EC was quantified by counting the number of Dil-ac-LDL-labeled cells incorporated into the tubes in 18 fields (×200) per group.

**Data analysis and statistics.** The results are expressed as means ± SE. P < 0.05 was considered significant. To evaluate the significance of differences in vessel density between stimulated and unstimulated sides of the TA muscle, a paired t-test (unstimulated vs. stimulated) and one-way analysis of variance were performed. Significant differences between groups were further investigated using a post hoc Tukey’s test. All data were tested to assure the normality of distribution requirement was met.

**RESULTS**

Attenuated therapeutic efficacy of SS/Mcwi BMC transplantation on skeletal muscle angiogenesis induced by electrical stimulation. The effect of BMC injection on skeletal muscle vessel density was evaluated after 7 days of electrical stimulation. Figure 1B shows that PBS injection as well as the autologous transplantation of BMC derived from an SS/Mcwi rat did not restore skeletal muscle angiogenesis in SS/Mcwi rats after electrical stimulation. To investigate if genetic manipulations of the RAS would affect angiogenesis induced by stem cell therapy, whole BMC isolated from the SS-13BN/Mcwi consomic rats were injected into the stimulated leg of the SS/Mcwi rats. We have previously reported that the SS/Mcwi rat has an impaired regulation of the renin gene located on chromosome 13 (3, 8, 9). Consomic SS-13BN/Mcwi rats are compatible BMC donors since they have the entire SS/Mcwi genetic background with the exception of the chromosome 13 from the normotensive BN rat, which contains a functioning renin gene. In contrast to the BMC derived from the SS/Mcwi rats, BMC
derived from the SS-13BN rats promoted a significant increase in the TA muscle vessel density of the SS/Mcwi rats after 7 days of electrical stimulation. To test the hypothesis that the low ANG II levels in the SS/Mcwi could impact the ability of the bone marrow stem cell therapy to restore skeletal muscle angiogenesis during electrical stimulation, BMC were isolated from SS/Mcwi rats that had been infused with low doses of ANG II for 7 days and then injected into the stimulated TA of another group of SS/Mcwi rats. As shown in Fig. 1B the BMC derived from the ANG II-infused SS/Mcwi rat restored the angiogenic response in the TA muscle after 7 days of electrical stimulation. Figure 2 confirms that the BMC labeled with PKH-67 dye actively incorporated into the skeletal muscle vasculature (Fig. 2A) and many of the labeled cells were colocalized with capillaries (Fig. 2B).

**In vivo ANG II infusion increases VEGF protein levels in SS/Mcwi whole BMC.** To evaluate the effect of ANG II infusion on VEGF protein levels in the BMC, a quantitative Western blot analysis with densitometry was performed. Figure 3 shows that low-dose ANG II infusion significantly increased VEGF protein levels in the BMC of SS/Mcwi rats.

**Bone marrow-derived endothelial characterization.** After 7 days in culture BM-EC exhibited cobblestone morphology, which is typical for confluent EC. The endothelial phenotype was further confirmed by the expression of CD31 (Fig. 4A), the ability to react with BS-1 lectin (Fig. 4B) and to uptake ac-LDL (Fig. 4C). Fluorescent microscopy identified double positive cells as BM-EC (Fig. 4D).

**In vivo ANG II infusion improves SS/Mcwi BM-EC function potentiating HUVEC tube network formation.** A Matrigel tube formation assay was performed to investigate whether the in vivo restoration of ANG II levels would affect the ability of SS/Mcwi derived BM-EC to integrate into vascular structures. As shown in Fig. 5A, HUVEC form vascular structures on Matrigel, and the BM-EC actively incorporate into the DAPI-labeled HUVEC network structure (Fig. 5B). Although the incorporation rate was not significantly higher in the BM-EC derived from SS/Mcwi rats infused with ANG II (Fig. 5E), the cells derived from ANG II infused SS/Mcwi rats significantly increased the total length of the vascular network structure formed by HUVEC compared with the BM-EC derived from SS/Mcwi infused with saline (Fig. 5, C, D, and F).

**DISCUSSION**

This study tested the hypothesis that low RAS activity in SS/Mcwi rats would impair BM-EC function and consequently angiogenic efficacy of bone marrow-derived stem cell therapy. Our results, using the SS rat, the consomic SS-13BN/Mcwi with a normal RAS and SS rats with ANG II replacement therapy support this hypothesis.

In the past decade studies have shown that the bone marrow derived EPC actively participate in adult angiogenesis. These studies have received a great deal of attention due to their potential for cell-based clinical therapies in many pathologies. A major therapeutic limitation is that EPC levels and function are reduced in patients with high cardiovascular risk, and angiogenesis efficacy in these patients is significantly impaired...
after autologous stem cell transplantation (22, 48, 58). Among the individual risk factors investigated, hypertension emerged as the strongest predictor of EPC migratory impairment (58). In addition, EPC senescence has been reported to be accelerated in both experimental hypertensive rats and patients with essential hypertension (26). Nevertheless, the mechanisms involved in the EPC dysfunction, as well as its time course during hypertension, it is not clear. Thus, a major remaining challenge is to elucidate the mechanisms underlying the EPC dysfunction and to develop new strategies to improve EPC properties and consequently the revascularization outcome after stem cell transplantation.

In the present study we used the SS/Mcwi rats as an animal model for low-renin hypertension and microvascular rarefaction (7) to explore the role of the RAS in bone marrow-derived EPC-mediated angiogenesis. The normotensive SS-13BN/Mcwi consomic served as a source of control cells for transplant and allowed us to investigate the hypothesis that impairments in the RAS may be responsible for the inability of SS/Mcwi cells to normalize angiogenesis.

The effect of ANG II on EPC function is controversial. Some studies have shown that ANG II diminishes telomerase activity of circulating EPC and accelerates the onset of EPC senescence through an increase in oxidative stress (24). In contrast, studies have shown that ANG II increases NO production, inhibits apoptosis, and enhances adhesion potential of bone marrow-derived EPC (62). Although ANG II inhibited EPC proliferation in one study, it enhanced VEGF-induced EPC proliferation in another (24, 25). Thus, further studies are necessary to define the mechanisms by which ANG II stimulates or inhibits EPC proliferation, adhesion, homing, and differentiation, as well as its impact in the angiogenesis stem cell therapy.

We have previously shown that changes in the ANG II levels, as well as genetic manipulation of the renin gene, have profound effects on skeletal muscle angiogenesis induced by exercise or electrical stimulation (1–3, 9–11, 41). In the present study we have shown that autologous bone marrow stem cell implantation failed to restore skeletal muscle angiogenesis induced by electrical stimulation in SS/Mcwi rats. We hypothesized that the failure in the bone marrow stem cell therapy to restore angiogenesis during electrical stimulation is related to a low RAS activity present in SS/Mcwi rats. Although RAS activity was not measured in the present study, we have previously demonstrated the renin activity profile of BN, SS/Mcwi, and consomic SS-13BN, as well as its correlation with the angiogenic phenotype in several studies (3, 8, 9). In contrast to the BN and SS-13BN rats, the renin activity was significantly lower in SS/Mcwi rats and is associated with an impaired angiogenesis response after electrical stimulation (8, 9). ANG II infusion at a very low dose, as well as the transfer of the entire chromosome 13 or even a small region of the chromosome 13 containing the renin gene, from the normotensive BN rat into the SS/Mcwi genetic background, completely restores the angiogenesis response after electrical stimulation (3, 9). Furthermore, the angiogenic response is inhibited in SS-13BN rats when the rats are treated with high-salt diet or the AT1 receptor blocker Losartan (9). These studies suggest that RAS has an important role in skeletal muscle angiogenesis; however, the mechanisms underlying the impaired RAS activation in the SS/Mcwi and the regulation of the capillary growth process are not totally understood. In the present study we show that low RAS activity observed in SS/Mcwi rats is associated with an impaired BM-EC function that may lead to failure in the angiogenesis process after stem cell therapy.
A significant increase in skeletal muscle vessel density was observed after BMC implantation derived from the SS-13BN rat, suggesting that the dysregulation of genes in the chromosome 13 may lead to an impaired bone marrow derived BM-EC function. Although there are many genes in the chromosome 13, previous studies from our laboratory indicate the renin gene as a strong candidate for the control of the angiogenesis response after electrical stimulation (3, 9). Using marker-assisted breeding four chromosome 13 congenic strains were developed that included or excluded the renin locus from the BN rat into the SS/Mcwi background (9). Microvessel density was markedly increased after stimulation in congenic strains that contained the renin gene from the BN rat and suppressed in control strains that carried regions of the BN genome just above or just below the renin gene. Renin is a key enzyme in the production of ANG II, and indeed low-dose ANG II infusion also restored angiogenesis induced by electrical stimulation in SS/Mcwi rats (9). The present study suggests that the restoration of ANG II levels in SS/Mcwi rats we improved the BM-EC function in vitro as measured by the vascular tube formation assay.

The slight increase in the VEGF protein levels in the BMC after ANG II infusion may improve BM-EC function. VEGF has been reported to increase the mobilization, proliferation, as well as the survival of the EPC (5, 57). In past few years, the use of stem cells overexpressing single or multiple growth factors is gaining popularity as therapeutic agents for neovascularization of ischemic tissue (43). Transplantation of the mesenchymal stem cells overexpressing VEGF in combination with cytokine therapy has shown superior BMC mobilization and EC proliferation, more pronounced angiogenic response, reduced tissue damage, and improved cardiac function (59). In vitro studies suggest that ANG II increases VEGF mRNA expression and protein synthesis in mesenchymal stem cells throughout activation of the AT1 receptor (49), and it is a potent stimulator of VEGF-induced proliferation and network formation of human EPC (25) via upregulation of VEGF receptor kinase domain-containing receptor (KDR). In agreement with these studies, we have demonstrated that systemic low-dose ANG II infusion increased VEGF protein level in the BMC of SS/Mcwi rats and also improved the ability of BM-EC to work in concert with the mature EC to form vascular tubes on Matrigel. Studies have shown that the efficiency of neovascularization may not solely be attributable to the incorporation of EPC in newly formed vessels but may also be influenced by...
the release of proangiogenic factors in a paracrine manner (61). The local release of growth factors by EPC may influence the classical process of angiogenesis, including the proliferation and migration as well as survival of mature EC (16). Therefore, the increase in skeletal muscle angiogenesis in vivo may be a result of the EPC paracrine effects in addition to the physical incorporation of EPC into newly formed capillaries.

Recent studies have shown that ANG II increases NO production, inhibits apoptosis, and enhances adhesion potential of bone marrow-derived EPC (62). This study shows that ANG II signaling through AT1 receptors activated PI3K/Akt pathways and significantly decreased EPC apoptosis. Although we haven’t investigated the effect of ANG II on EPC survival in vivo it is possible that the increase in angiogenesis may be related to a greater survival of the transplanted EPC-derived BMC. We have previously shown that low-dose ANG II infusion in animals fed a high-salt diet significantly reduced vascular EC apoptosis and restored skeletal muscle angiogenesis induced by electrical stimulation (10). Since both EPC and mature EC share many of common features including the expression of endothelial-specific markers, VEGFR-2, Tie-1, Tie-2, and VE-cadherin (4, 27, 39, 60), the ability to uptake ac-LDL and to form vascular structure in vitro (27, 45), it is likely that EPC function is affected by common EC mediators. The present study suggests that besides the classic ANG II angiogenic mechanisms, including increase in mature EC survival, migration, adhesion, and proliferation,(6, 15, 23, 31, 52) ANG II infusion may also improve BM-EC function and, as such, may impact BM-EC biology and consequently the organ vascularization and the regeneration process induced by bone marrow stem cell based therapy. Further studies using ANG II receptor blockers would support this hypothesis.

ACKNOWLEDGMENTS
The authors thank Justin Friske for expert technical assistance.

GRANTS
This work was supported by National Institutes of Health Grants Support NIH P01-HL-082798; N01-HV-28182.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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