ACE2/Ang-(1–7)/Mas axis stimulates vascular repair-relevant functions of CD34+ cells

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1Department of Pharmaceutical Sciences, North Dakota State University, Fargo, North Dakota; 2Department of Pathophysiology, College of Basic Medical Science, Jilin University, Changchun, Jilin, China; 3Department of Chemistry, University of Florida, Gainesville, Florida; and 4Department of Physiology and Functional Genomics, College of Medicine, University of Florida, Gainesville, Florida

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Singh N, Joshi S, Guo L, Baker MB, Li Y, Castellano RK, Raizada MK, Jarajapu YP. ACE2/Ang-(1–7)/Mas axis stimulates vascular repair-relevant functions of CD34+ cells. Am J Physiol Heart Circ Physiol 309: H1697–H1707, 2015. First published September 18, 2015; doi:10.1152/ajpheart.00854.2014.—CD34+ stem/progenitor cells have been identified as a promising cell population for the autologous cell-based therapies in patients with cardiovascular disease. The counter-regulatory axes of renin angiotensin system, angiotensin converting enzyme (ACE)/Ang II/angiotensin type 1 (AT1) receptor and ACE2/Ang-(1–7)/Mas receptor, play an important role in the cardiovascular repair. This study evaluated the expression and vascular repair-relevant functions of these two pathways in human CD34+ cells. CD34+ cells were isolated from peripheral blood mononuclear cells (MNCs), obtained from healthy volunteers. Expression of ACE, ACE2, AT1, and angiotensin type 2 and Mas receptors were determined. Effects of Ang II, Ang-(1–7), Norleu3-Ang-(1–7), and ACE2 activators, xanthenone (XNT) and diminazene acetate (DIZE) on proliferation, migration, and adhesion of CD34+ cells were evaluated. ACE2 and Mas were relatively highly expressed in CD34+ cells compared with MNCs. Ang-(1–7) or its analog, Norleu3-Ang-(1–7), stimulated proliferation of CD34+ cells that was associated with decrease in phosphatase and tensin homologue deleted on chromosome 10 levels and was inhibited by triciribin, an AKT inhibitor. Migration of CD34+ cells was enhanced by Ang-(1–7) or Norleu3-Ang-(1–7) that was decreased by a Rho-kinase inhibitor, Y-27632. In the presence of Ang II, XNT or DIZE enhanced proliferation and migration that were blocked by DX-600, an ACE2 inhibitor. Treatment of MNCs with Ang II, before the isolation of CD34+ cells, attenuated the proliferation and migration to stromal derived factor-1a. This attenuation was reversed by apocynin, an NADPH oxidase inhibitor. Adhesion of MNCs or CD34+ cells to fibronectin was enhanced by Ang II and was unaffected by Ang-(1–7). This study suggests that ACE2/Ang-(1–7)/Mas pathway stimulates functions of CD34+ cells that are cardiovascular protective, whereas Ang II attenuates these functions by acting on MNCs. These findings imply that activation of ACE2/Ang-(1–7)/Mas axis is a promising approach for enhancing reparative outcomes of cell-based therapies. CD34+ cells; ACE-2; Ang-(1–7); Mas receptor; migration; proliferation

NEW & NOTEWORTHY

Angiotensin-(1–7) or its analog NorLeu3-Ang-(1–7) and the putative angiotensin converting enzyme (ACE)2 activators, xanthenone and diminazene acetate, stimulate migration and proliferation - functional signatures of vasoreparative potential - of human CD34+ cells. Angiotensin II, by stimulating the generation of reactive oxygen species from mononuclear cells, attenuates the functions of CD34+ cells.

CD34, a SIALOMUCIN-LIKE LIGAND for L-selectin, is long known to be a common cell-surface marker to many types of hematopoietic progenitor cells (7). Adult CD34+ cells are bone marrow-derived multipotent stem cells and are capable of reconstituting hematopoietic systems (5). This is a major cell type for individuals needing hematopoietic reconstitution following chemotherapy or radiation therapy (9). Interestingly, CD34 is robustly expressed in small capillaries and in tissues that support early vascular development (48, 53). However, the role of CD34+ cells in postnatal vasculogenesis was not known until recently. The groundbreaking studies by Asahara et al. (3) provided compelling evidence for a novel role of CD34+ cells in the vascularization of ischemic tissue. Therefore, CD34+ cells were quite often designated as endothelial progenitor cells. It has now been well documented that these cells in response to hypoxia-regulated factors such as stromal-derived factor-1 (SDF) and VEGF proliferate and migrate to the areas of ischemia, and accelerate the vascular repair thus preventing tissue damage (26). Autologous cell therapy using CD34+ cell population has now emerged as a promising approach for the treatment of myocardial ischemic disease (32).

Classically, renin angiotensin system (RAS) consists of angiotensin converting enzyme (ACE), its product angiotensin (Ang) II and receptors for Ang II, angiotensin type 1 (AT1), and angiotensin type 2 (AT2) receptors. RAS has been expanded extensively in the recent years with the identification of ACE2, a monocarboxy peptidase that generates Ang-(1–7) from Ang II and the identification of the receptor, Mas, which mediates the biological actions of Ang-(1–7) (44). Although Ang II produces hypertensive, pro-oxidative, hypertrophic and pro-fibrotic effects in cardiovascular system, Ang-(1–7) elicits counter-regulatory effects on ACE/Ang II pathway by producing vasodilatory, antihypertensive, antiapoptotic, antifibrotic, and anti-thrombotic effects (11, 23).

Local or tissue expression of ACE and ACE2 systems has been identified and was shown to have paracrine, autocrine, and intracrine effects (33, 37). Evidence for the local RAS in bone marrow has been provided by studies that have shown hematopoietic functions of angiotensin peptides in human and murine bone marrow cells (39, 43). With respect to the functions of reparative and regenerative functions of CD34+ cells, role of local RAS is yet to be investigated. We have recently reported that ACE2 and Mas receptor are expressed in these...
cells and that Ang-(1–7) support migratory function (24). In this study we have characterized the expression of ACE and ACE2 axes in total mononuclear cells, Lineage-negative (Lin−) cells that are enriched for primitive stem/progenitor cells and CD34+ cells. Effects of Ang II, Ang-(1–7) or its analog NorLeu3-Ang-(1–7), and the putative ACE2 activators dimazene acetate (DIZE) (45) and xanthenone (XNT) (12, 15) were evaluated on proliferation, migration, and adhesion, which are in vitro indicators of vasoparative functions of CD34+ cells.

MATERIALS & METHODS

Characteristics of subjects. This study was approved by Institutional Biosafety Committee of North Dakota State University (protocol No. B12017). The work described has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Human leucocyte samples were obtained from United Blood Services (Fargo, ND). Healthy volunteers included both males and females of age ranging from 48 to 75 years. All volunteers are Caucasians. Leucocytes were collected in chambers of Leucoreduction system (LRS chambers) following apheresis carried out by using Trima Accel system (80440).

Isolation of cells. CD34+ cells were isolated from leucocytes as described before (25). Peripheral blood mononuclear cells (MNCs) were separated from total leucocytes by gradient centrifugation using ficoll (Ficoll-Paque; GE Healthcare Biosciences). Plasma was completely excluded from the cell fraction by a series of washings using PBS with 2% FBS and 1 mM EDTA and centrifugation at 120 g. These cells were enriched for lineage negative (Lin−) cells by using a negative selection kit (StemCell Technologies) as per supplier’s instructions. Lin− cells were then enriched for CD34+ cells by using an immunomagnetic separation kit (EasySep, Human CD34 positive selection kit; StemCell Technologies) as per the manufacturer’s instructions. The purity of enriched CD34+ cells was assessed by flow cytometry. Cells were stained by incubating with anti-CD45 and anti-CD34 antibodies (R&D systems) or isotype control antibodies in the presence of FcR blocking reagent (Milteny Biotech) for 45 min at 4°C. Cells were analyzed by a cytometer (C6 Accuri, BD). The purity ranged from 86% to 92%, when evaluated randomly during this study.

MNCs, Lin− cells, or CD34+ cells were used for different experimental protocols. Freshly isolated cells were either used for assays or preserved by snap-freezing cell pellets in liquid nitrogen and storing at −80°C for later use. When needed, freshly isolated Lin− or CD34+ cells were plated in U-bottom 96-well plates (Nunc) in StemSpan or MNCs, Lin− cells, and CD34+ cells by Western blot analysis. Briefly, equal concentrations of proteins from whole cell lysates were resolved by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane and stained with primary antibodies, mouse anti-human ACE (GTX11737; Genetex), rabbit anti-human ACE2 (sc-20998; Santa Cruz Biotechnology), mouse anti-human Mas1, or rabbit anti-human AT1 (ab124734; Abcam). β-Tubulin was used as a loading control and stained with rabbit anti-human antibody (Santa Cruz BioTechnology). IgG-HRP conjugated, goat anti-mouse, or goat anti-rabbit secondary antibodies were obtained from Bio-Rad. Blots were processed by using ECL Prime (GE Healthcare).

Recent studies (4, 21) have questioned the specificity of commercially used antibodies of mouse or rat AT1 receptor; therefore, we sought to confirm the specificity of the antibody used in the current studies. This was accomplished by RNA silencing approach by using small interference RNA (siRNA) (Catalog No. sc-29750; Santa Cruz BioTechnology). MNCs were transfected with 5 μM siRNA duplex with transfection medium as per the manufacturer’s protocol for 72 h. MNCs treated with serum-free medium or scrambled siRNA (Catalog No. sc-37007) served as a control and as a negative control, respectively. Expression of AT1 receptor mRNA and protein were quantified as described above by real-time PCR and by Western blotting, respectively.

Flow cytometric analysis. Surface expression of Mas receptor in CD34+ cells was evaluated by flow cytometry using two different protocols. The first method was performed by staining the CD34+ cells (1 × 10⁵) with primary rabbit anti-MAS1 antibody (NEL1531; Novus Biologicals) for 45 min at room temperature after blocking with FcR blocking reagent (Milteny Biotech). The cells were then washed three times with washing buffer, and a Dylight 488 anti-rabbit secondary antibody (Vector laboratories) was added to cells for 30 min at room temperature. The cells were analyzed for green fluorescence by using Accuri C6 flow cytometer (BD Biosciences). For the second method, a fluorescent self-conjugated MAS1 antibody was used to detect the receptor presence on CD34+ cells. The conjugation was performed by using Zenon-APC Rabbit IgG labelling kit (Life Technologies) as per the manufacturer’s protocol. Briefly, 5 μl of the Zenon rabbit IgG labelling reagent was added to 1 μg of primary rabbit anti-MAS1

Table 1. List of primer sets used for the real-time PCR studies

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Primer Sequence</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Angiotensin converting enzyme 2</td>
<td>5'-CATTGGAAAGCATGTTGATCCTT-3'</td>
<td>5'-GAGCTATGCTGGAGATTCTCA-3'</td>
<td></td>
</tr>
<tr>
<td>Angiotensin type 1 receptor</td>
<td>5'-AAGGCAATGCTGTCTTAGCA-3'</td>
<td>5'-CTGGAATTTCAACGTGCCATTG-3'</td>
<td></td>
</tr>
<tr>
<td>Angiotensin type 2 receptor</td>
<td>5'-GGCTGCTGAAACATGCTGGA-3'</td>
<td>5'-GTAAACTCAGCCCAAGGAGG-3'</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-GACAGATTGGAGAACAGTATTACT-3'</td>
<td>5'-GATGCCACATGTGCTGGAGGT-3'</td>
<td></td>
</tr>
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AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00854.2014 • www.ajpheart.org
antibody (Novus Biologicals) antibody in PBS and incubated for 5 min at room temperature. Then, 5 μl of the Zenon blocking reagent was added to the reaction mixture and incubated for 5 min at room temperature. The complexes were then applied immediately to CD34⁺ cell suspension in staining buffer treated with FcR blocking reagent and incubated for 45 min at 4°C. The cells were analyzed for APC fluorescence by flow cytometry.

To assess the expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), an intracellular protein, CD34⁺ cells were treated with FcR blocking reagent, and then fixed and permeabilized with BD Cytofix/Perm Reagent (BD Biosciences). Cells were then stained with Alexa Fluor 647 Mouse anti human-PTEN (BD PhosFlow) or isotype control antibody for 45 min at 4°C. Cells were analyzed by flow cytometry. Effect of treatments on PTEN levels was determined by enumerating PTEN-expressing cells and by quantifying mean fluorescence intensity.

**ACE2 activity assay.** ACE-2 activity was determined by using the intramolecularly quenched synthetic ACE2 specific substrate 7-Mca-YVADAPK(Dnp) (R&D systems) (20). Protein (10 μg) from cell lysates was used for the assay in a buffer containing 50 mM 4-morpholineethanesulphonic acid, 300 mM NaCl, 10 μM ZnCl₂, and 0.01% Triton X-100 at pH 6.5. Reaction was initiated by the addition of 50 μM substrate, and the fluorescence was monitored for 3 h by using a plate reader (SpectraMax M5; Molecular Devices) at an excitation wavelength 320 nm and emission wavelength 405 nm. ACE 2 activity was defined as ACE2 inhibitor (MLN4760, 10 μM)-sensitive fluorescence and was expressed as percentage of total fluorescence.

Fig. 1. Comparative expression of mRNA and protein of angiotensin converting enzyme (ACE), ACE2, MAS1, Ang II type 1 receptor (AT₁R), and Ang II type 2 receptor (AT₂R) in human mononuclear cells (MNCs), lineage-negative cells (Lin⁻ cells), and CD34⁺ cells. A: expression of mRNA was represented by ΔCt values relative to the expression of β-actin (n = 5 to 7). B: representative dot plots of Mas receptor expression in CD34⁺ cells detected by using primary and a Dylight 488-labeled secondary antibody (left) or by using Zenon-APC labeling kit of primary antibody (right) (n = 4). Shown were CD34⁺ cell number versus fluorescence intensity, and the rightward shift indicates the surface expression of Mas receptor. C: specificity of AT₁R antibody: treatment of MNCs with small interference RNA (siRNA) resulted in robust decrease in the expression of mRNA or protein of AT₁R. Shown were representative Western blot of AT₁R in MNCs, untreated control, and siRNA- or scramble-siRNA-treated. Levels of mRNA were expressed relative to the untreated control. Expressions of mRNA or protein following treatments were compared by 1-way ANOVA (n = 6). D: representative Western blots of ACE, ACE2, and AT₁R proteins in human CD34⁺ cells in comparison with the expression in MNCs. Expression of proteins was quantified relative to the β-tubulin and was lower in CD34⁺ cells compared with MNCs (n = 6). E: ACE2 activity in MNCs and CD34⁺ cells: activity was defined as MLN4760-inhibitable fluorescence of the fluorogenic substrate and was expressed as percentage of total fluorescence. Activity observed in MNCs was significantly higher than the activity in CD34⁺ cells (n = 6; t-test).
Proliferation assay. Proliferation of CD34+ cells was evaluated by determining BrdU incorporation using a kit (Cell Proliferation ELISA; Roche Bioscience) as per the manufacturer’s instructions. The assay was performed by using 10,000 cells per condition, and each sample was tested in duplicate. Cells were plated in basal medium StemSpan (Stemcell Technologies) with or without drug treatments, and the proliferation was evaluated after 48 h. Absorbance was quantified by using Spectramax plate reader. Proliferation was expressed as fold increase relative to the effect of mitomycin (1 μM), which inhibits proliferation.

Migration assay. As described before (25), migration of CD34+ cells was evaluated by the QCM Chemotaxis cell migration assay kit (EMD Millipore) as per the manufacturer’s instructions. Assay was carried out by using 20,000 cells per treatment in a basal medium, HBSS (Bioportech), and each sample was tested in duplicate. Cells were allowed to migrate in response to the treatments for 5 h, and the response was expressed as arbitrary fluorescence units.

Adhesion assay. Adhesion assay was carried out as described in a previous study (27). Briefly, peripheral blood MNCs (1 million) or CD34+ cells (5 × 10^5) were suspended in EBM-2 medium supplemented with EGM-2MV SingleQuots and plated in each well of a 6-well or a 24-well plate, respectively. The plates were coated with fibronectin (Sigma) at least an hour before plating the cells. In case of MNCs, after 4 h of plating, the nonadherent cells were collected and were replated on another fibronectin-coated well. Cells were treated with 100 nM Ang II once a day for 4 days following plating on fibronectin. On day 4, nonadherent cells were removed by PBS wash before acquiring bright field images. In some experiments, adhesion was evaluated 4 h following replating of MNCs. In case of CD34+ cells, which are nonadherent by nature, treated with Ang II once a day for 4 days following plating on fibronectin. On day 4, nonadherent cells were removed by using PBS before acquiring bright field images of adhered cells. Images of adhered cells were obtained by using a 4× objective lens Olympus microscope. Adhered cells were enumerated by using ImageJ software (National Institutes of Health).

Drugs. Norleu’Angiotensin-(1–7), Angiotensin-(1–7), Angiotensin II, and A779 were obtained from Bachem Americas (Torrance, CA). Losartan and apocynin were purchased from Sigma (St. Louis, MO). DIZE and DX600 were purchased from LKT Laboratories (St. Paul, MN) and Anaspec (Fremont, CA), respectively. MLN-4760 was purchased from EMD Millipore. Xanthenone Derivative, 1-[[4-(dimethylamino)ethyl]amino]-4-(hydroxymethyl)-7-[[4-(methylphenoxy)sulfonyl]oxy]-9H-xanthene-9-one (XNT), and MLN were synthesized in the Core Synthesis Facility, North Dakota State University (Fargo, ND) according to the method reported by Archer et al. (2) with major modifications (see Supplemental Material).

Data analysis and statistics. Results were expressed as means ± SE, and n represents the number of donors used. Results were analyzed for statistical significance by using the software program GraphPad (GraphPad Prism). Either t-test or one-way ANOVA with Bonferroni’s post-test for multiple comparisons was used where appropriate. Power analysis was carried out post hoc by using Minitab software program, and all experiments are powered at 0.8 or higher.

RESULTS

Expression of ACE2/Mas is higher in lin− cells and CD34+ cells. Evaluation of the expression of RAS components by real-time PCR identified mRNA transcripts of ACE, ACE2, AT1R, AT2R, and Mas receptor in MNCs, Lin− cells, and CD34+ cells. The data were analyzed for comparing the expression of a member of RAS in three different cell populations. The expression of Mas was found to be higher (low ΔCt) in Lin− (P < 0.05) or CD34+ (P < 0.01) cells compared with MNCs (Fig. 1A). ACE2 expression showed similar trend as Mas, higher expression in Lin− or CD34+ (P < 0.01) cells compared with MNCs (Fig. 1A). In contrast, ACE expression is similar among all three different cell populations (Fig. 1A); however, the expression of AT1R or AT2R is not significantly different among different cell populations (Fig. 1A). In MNCs, expression of ACE is higher than ACE2 (P < 0.001). In CD34+ cells, expression of Mas is higher than AT1R or AT2R (P < 0.001). Furthermore, protein expression of ACE, ACE2, AT1, and Mas were confirmed by either flow cytometry (Fig. 1B), or by activity assay (Fig. 1E) or by Western blotting (Fig. 1, C and D) in MNCs and CD34+ cells. Specificity of AT1R antibody was confirmed by RNA silencing approach. Treatment with siRNA resulted in robust decrease in the mRNA expression of AT1R compared with the untreated control (P < 0.001) or the scramble-siRNA-treated cells (P < 0.005; n = 6).

Fig. 2. A: effect of Ang-(1–7), Norleu’Ang-(1–7), or ACE2 activators on proliferation of CD34+ cells. Norleu’Ang-(1–7) and Ang-(1–7) stimulated proliferation of CD34+ cells (n = 8). Proliferation induced by Norleu’Ang-(1–7) (100 nM) was inhibited by A779 (n = 8) or by combination of A779 and losartan (n = 5). Along similar lines, the effect of Ang-(1–7) was inhibited by A779 (n = 8), which was not affected by losartan, alone or in combination with A-779 (n = 8; 2-sample t-test). B: Ang II has no effect on proliferation. Diminazene aceturate (DIZE) and xanthenone (XNT) have stimulated proliferation in the presence of Ang II, and this response was blocked by DX-600 (n = 5; paired t-test).
In the siRNA-treated MNCs, detection of the AT$_1$R protein by the antibody, ab124734, was almost absent, whereas protein expression was apparent in the scramble-siRNA-treated cells, compared with the untreated control ($n = 6$) (Fig. 1C).

Western blotting and ACE2-activity assay indicated no correlation between protein or activity and mRNA levels. Despite the higher mRNA expression in CD34$^+$ cells, the protein expression of ACE, ACE2, and AT$_1$R were lower than those observed in MNCs (Fig. 1D). Consistent with this, ACE2 activity was higher in MNCs compared with CD34$^+$ cells ($P < 0.001$; $n = 6$) (Fig. 1E).

Activation of Mas receptor or ACE2 promotes proliferation of CD34$^+$ cells. Both Ang-(1–7) and Norleu$_3$-Ang-(1–7) stimulated proliferation of CD34$^+$ cells (Fig. 2A). The response was comparable with that produced by 100 nM SDF. The effect of Norleu$_3$-Ang-(1–7) was partially antagonized by 1 $\mu$M A779. Losartan (1 $\mu$M), an AT$_1$R antagonist, alone did not affect this response; however, to our surprise, with the combination of A779 and losartan, the effect was completely antagonized (Fig. 2A). The effect of Ang-(1–7) was sensitive to blockade by Mas receptor antagonist A779 and was not affected by losartan. In agreement with these findings activators of ACE2 stimulated proliferation in CD34$^+$ cells. In the presence of 100 nM Ang II, 100 nM DIZE or 100 nM XNT increased the proliferation of CD34$^+$ cells compared with Ang II alone (Fig. 2B). The effect of either XNT or DIZE was inhibited by an ACE2 blocker 1 $\mu$M DX600. Neither DIZE nor XNT affected proliferation in the absence of Ang II, a substrate for ACE2 (data not shown). Along the similar lines, Ang II alone did not affect the proliferation of CD34$^+$ cells (Fig. 2B).

PTEN has been shown to be a major regulator of human and murine hematopoietic stem cell functions (30). PTEN opposes the proliferation of human CD34$^+$ cells by negatively regulating phosphatidylinositol-3,4,5 trisphosphate/AKT signaling.
pathway (30). We have previously shown that proliferation and survival of CD34+ cells induced by Ang-(1–7) is phosphatidylinositol-3-kinase (PI3K)/Akt-dependent (24). In the presence of an AKT inhibitor, triciribin, Norleu3-Ang-(1–7)-induced proliferation was significantly inhibited ($P < 0.01$; $n = 5$) (Fig. 3A). We then checked the levels of PTEN in cells proliferating in response to Norleu3-Ang-(1–7). Flow cytometric evaluation of PTEN in CD34+ cells indicated significant decrease in the number of cells expressing PTEN-expressing cells ($P < 0.01$) as well as the mean fluorescence intensity ($P < 0.01$; $n = 9$) following treatment with Norleu3-Ang-(1–7) compared with the untreated (Fig. 3, B–D). Furthermore, the effects of DIZE or XNT were evaluated on PTEN levels in CD34+ cells. As predicted, both of these molecules produced significant decrease in the number of PTEN-expressing cells and the fluorescence intensity ($n = 7–9$) (Fig. 3, B–D).

Activation of Mas receptor or ACE2 promotes migration of CD34+ cells. Both Ang-(1–7) and Norleu3-Ang-(1–7) stimulated migration of CD34+ cells ($P < 0.05$; $n = 7–9$; Fig. 4A). However, the effects were not concentration dependent (not shown). The responses are comparable with that produced by SDF (Fig. 4A). Effects of both Ang-(1–7) and Norleu3-Ang-(1–7) were inhibited by A779 (Fig. 4A). XNT or DIZE, on their own, did not affect the migration of CD34+ cells (data not shown). In the presence of Ang II, migration was significantly enhanced by DIZE ($P < 0.01$; $n = 5$) or XNT ($P < 0.03$; $n = 5$) (Fig. 4B). Ang II alone did not affect migratory response. We further observed that migration induced by Norleu3-Ang-(1–7) was significantly inhibited by Rho-kinase (ROCK) inhibitor Y-27632 (10 μM; $P < 0.02$; $n = 6$) (Fig. 4C). Similar blockade was also observed in SDF-induced migration ($P < 0.01$; $n = 6$; Fig. 4C).

Ang II attenuates migration and proliferation of CD34+ cells by acting on MNCs. As shown above, Ang II, on its own, did not affect basal migration or proliferation of CD34+ cells (Figs. 2 and 3). We then tested whether Ang II could modulate CD34+ cell functions indirectly via acting on MNCs. This was accomplished by the treatment of MNCs with 100 nM Ang II, as described above, followed by isolation of CD34+ cells. Pretreatment with Ang II before isolation significantly attenuated SDF-mediated migration (Fig. 5A) or proliferation (Fig. 5B) in CD34+ cells. Ang II is known to activate NADPH-oxidase and to increase the generation of reactive oxygen species (ROS), such as superoxide, (35) which could in turn impair physiological functions of CD34+ cells including migration (25). Therefore, we tested whether the impaired migration and proliferation were due to the activation of NADPH-oxidase by Ang II in MNCs. This was achieved by treatment of MNCs with apocynin (300 μM), an inhibitor of NADPH-oxidase, during Ang II treatment. Treatment with apocynin reversed Ang II-induced impairment in SDF-migration (Fig. 5A) or proliferation (Fig. 5B) in CD34+ cells. To our surprise, in the presence of apocynin, basal and stimulated migration.

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Fig. 4. Effects of Ang-(1–7), Norleu3-Ang-(1–7), or ACE2 activators on migration of CD34+ cells. A: migration was expressed as percentage of response observed in untreated/control cells. Migration induced by 100 nM Ang-(1–7) or Norleu3-Ang-(1–7) was inhibited by A779 ($n = 5–9$; 1-way ANOVA). B: ACE2 activators, DIZE or XNT (100 nM), have stimulated migration in the presence of Ang II ($n = 5$; paired $t$-test). C: migration induced by Norleu3-Ang-(1–7) or stromal derived factor-1α (SDF) is Rho-kinase-dependent: Rho-kinase inhibitor Y-27632 (10 μM) did not affect basal migration on its own. Migration induced by SDF or Norleu3-Ang-(1–7) ($n = 6$; 2-sample $t$-test) was inhibited by Y-27632.
was increased in Ang II-pretreated cells, compared with the responses in cells-treated by apocynin only.

Ang II promotes adhesion of both MNCs and CD34⁺ cells. Finally, we tested whether Ang II or Ang-(1–7) alters the adhesion of MNCs or CD34⁺ cells. We tested the adhesion of MNCs or CD34⁺ cells to fibronectin, an extracellular matrix protein extensively used for studying vasoreparative functions of stem/progenitor cells. The integrin αβ₁ is known to mediate CD34⁺ cell adhesion to endothelial cells and to extracellular matrix proteins such as fibronectin (28, 52). Ang-(1–7) did not modify adhesion of CD34⁺ cells or MNCs on fibronectin (data not shown). Ang II increased the adhesion of MNCs to fibronectin following treatment for 4 days (P < 0.05; n = 6) of plating, compared with the untreated cells (Fig. 6A). Interestingly, adhesion of CD34⁺ cells was also enhanced by Ang II, when evaluated after 4-day treatment (P < 0.04 vs. untreated) (Fig. 6B).

DISCUSSION

This study reports several novel findings. ACE2/Mas expression is higher in primitive Lin⁻ or CD34⁺ cells compared with MNCs. Norleu³-Ang-(1–7) is as potent as Ang-(1–7) in inducing migration or proliferation in human CD34⁺ cells. Activation of MNCs by Ang II decreased proliferation and migration of CD34⁺ cells most likely by stimulating the generation of ROS by NADPH-oxidase. Adhesion of both MNCs and CD34⁺ cells was enhanced by Ang II. Thus, ACE2/Ang-(1–7) pathway produces vascular repair-relevant functions of CD34⁺ cells, whereas Ang II attenuates these functions indirectly by acting on MNCs.

Local RAS in CD34⁺ cells. This is the first study to evaluate the expression of local RAS in human CD34⁺ cells at mRNA and protein levels. CD34⁺ cells are primitive cells and are enriched in Lin⁻ cells. According to the present study, among all the five members of RAS, gene expression of Mas and ACE2 are higher in these primitive cells that have reparative functions, compared with the expression in MNCs, a fully differentiated cell population. On the other hand, ACE is expressed to a similar extent in both primitive and differenti-
pathway, which in turn regulates HSC proliferation and differentiation (56). Our findings suggested that Norleu³-Ang-(1–7)-induced proliferation was associated with a decrease in PTEN levels. A decrease in PTEN enhances accumulation of AKT, which promotes proliferation and self-renewal without inducing leukemic response, whereas complete deficiency of PTEN increases the risk for leukemia (54, 56). Thus PTEN is cell-autonomously required for the maintenance of stem/progenitor cells. The current study suggests that activation of Mas receptor does not induce leukemic response in human CD34⁺ cells, and thus modification of CD34⁺ cell functions by Ang-(1–7) or by ACE2 activators for cell-based therapies would most likely be free from myeloproliferative side effects.

Migration to the areas of ischemia is an important property of CD34⁺ cells that determines the reparative function of these cells. This function is modulated mainly by hypoxia-regulated factors such as SDF and VEGF (34). Current study shows that activation of ACE2 or Mas receptor induces migration of these cells that is comparable with the response produced by SDF. Effects of both Ang-(1–7) and Norleu³-Ang-(1–7) on migration were mediated by the activation of Mas receptor. This is in contrast with the effect on proliferation, which was partly mediated by AT₁ receptor (see above). This study further confirmed the beneficial effects of putative ACE2 activators, XNT and DIZE, on migration of CD34⁺ cells, and the presence of Ang II is necessary for their effects. Importantly, the effects were sensitive to blockade by a known ACE2 inhibitor, DX600 (22). ACE2 activation by these novel molecules has been confirmed in several in vitro and in vivo studies (12, 14, 38, 45, 49), whereas a recent study proposed ACE2-independent effects on cardiovascular hemodynamics (18). The in vitro experimental approach that was used in the current study, and the use of selective blocker of ACE2 confirms that the effects produced by these molecules in CD34⁺ cells are ACE2 dependent. Furthermore, the present study shows that migration induced by Mas receptor activation was partially sensitive to blockade by ROCK inhibitor Y-27632. ROCK promotes migration of cells by actin polymerization (46), which is the mediator of migratory response to SDF (51), and other growth factors, such as VEGF and PDGF (17, 55). Sensitivity of Mas-receptor-mediated migration to

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**Fig. 6.** Ang II increases adhesion of MNCs and CD34⁺ cells. A: representative bright field images of MNCs adhered to fibronectin with or without 100 nM Ang II treatment, and the bar graph depicting the effect of Ang II. Number of cells adhered were higher compared with control following Ang II treatment (n = 6). B: representative bright field images of CD34⁺ cells adhered to fibronectin with or without 100 nM Ang II treatment, and the bar graph depicting the effect of Ang II. Number of cells adhered were increased by Ang II treatment compared with control (n = 6).
the ROCK blockade suggests that Mas receptor, in part, acts through mechanisms that are activated by growth factors in CD34+ cells. However, involvement of alternative pathways in the response mediated by Norleuc1-Ang-(1–7) cannot be ruled out since the blockade by Y-27632 is partial.

**Ang II attenuates CD34+ cell functions by acting on MNCs.** In the current study, Ang II did not affect the proliferation or migration of CD34+ cells in vitro, although the mRNA transcript and protein for AT1 receptor were identified. Earlier studies have shown evidence for hematopoietic functions of Ang II in murine and human primitive progenitor cells (43). In vitro treatment with Ang II robustly enhanced differentiation of human CD34+ cells to Colony Forming Units via activating AT1 receptor; however, these studies did not evaluate proliferation without differentiation.

This study further evaluated the indirect effects of Ang II on the functions of CD34+ cells by activating other cell types. Ang II is known to modulate functions of different subsets of mononuclear leukocytes and lymphocytes via activating AT1 receptor and largely by inducing NADPH-dependent generation of ROS and oxidative stress (13). Reparative functions of CD34+ cells were shown to be attenuated by increased oxidative stress (25). In the current study, treatment of the whole MNC population with Ang II before isolation of CD34+ cells resulted in significant attenuation of proliferation and migration. Given the fact that CD34+ cells circulate with several of MNCs in the near vicinity, sustained elevation in the circulating Ang II would attenuate CD34+ reparative functions by increasing local oxidative stress in MNC-dependent mechanism. Although this study focused at the interaction between MNCs and CD34+ cells, the role of other inflammatory cells that are reactive to the Ang II cannot be ruled out and would likely amplify the attenuation of CD34+ cell functions.

Taken together, findings from studies involving apocynin and ROCK inhibitor imply either no interaction between ROS and RhoA/ROCK pathway or negative modulation of RhoA/ROCK activation by ROS. Inhibiting ROS increased migration of CD34+ cells, which is consistent with our previous study (25). Another study in monocytes showed compelling evidence for the attenuation of RhoA/ROCK activation and decreased migration by ROS (1). Therefore, in the current study, NADPH-derived ROS attenuated CD34+ cells migration very likely via attenuating ROCK activation, which is in contrast with several studies in vascular preparations that have consistently shown that ROS activates RhoA/ROCK pathway leading to increased vascular smooth muscle contraction (31, 35).

Adhesion is another important characteristic of CD34+ cells, which determines homing of cells to the areas needing vascular repair (29, 57). We have tested the property of adhesion on fibronectin because it is a ligand for the integrin α4β1, which is highly expressed in CD34+ cells (29). Fibronectin is highly expressed on proliferating endothelial cells (10) and in endothelial cells very early following vascular injury (8). Thus the selective expression of fibronectin in the areas of injury promotes homing of vasoreparative cells and accelerates vascular or tissue repair. CD34+ cells use α4 integrin to adhere to fibronectin-producing endothelium that facilitates extravasation of cells into damaged/inflamed tissue (16, 36). Although Ang-(1–7) did not have any effect on adhesion of CD34+ cells or MNCs, Ang II enhanced adhesion of both MNCs and CD34+ cells, likely by functional modification of integrins.

Although this study did not characterize functional modification of integrins that were specifically affected by Ang II, we did observe that the effect of Ang II could be reversed by inhibiting ROS generation. Inhibition of NADPH oxidase by apocynin reversed Ang II-mediated increase in the adhesion of MNCs or CD34+ cells, suggesting that Ang II-stimulated ROS generation increased the adhesion of cells, likely by modifying the integrin-fibronectin interactions. Unlike migration and proliferation, adhesion was enhanced by direct actions of Ang II on CD34+ cells. It is important to note that adhesion assay involved treatment of Ang II for a longer period, once a day for 4 days, and that the expression of AT1 receptor is very low. Although the adhesion of CD34+ cells to fibronectin is beneficial for vascular repair, adhesion of MNCs will not promote vascular repair and may even attenuate this process (28, 47). These findings are in agreement with the previous report that MNCs from hypertensive patients or those activated by Ang II adhere more compared with the MNCs from healthy individuals (47).

**Conclusions**

Overall, the current study shows that both cardiovascular protective and deleterious arms of RAS modulate the vasoreparative functions of CD34+ cells. ACE2/Ang-(1–7)/Mas axis activates vasoprotection-relevant functions of these cells. In contrast, ACE/Ang II/AT1 axis either directly or indirectly via acting on MNCs attenuates CD34+ cell functions. Therefore, relative expression of the two axes of local RAS in CD34+ cells could be a good measure of their vasoreparative functions. Along the similar lines, an imbalance toward increased expression of ACE/Ang II/AT1 axis would promote dysfunction of CD34+ cells and could be a prognostic marker for an increased risk for the development of cardiovascular disease.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

Author contributions: N.S., S.J., L.G., M.B., and Y.L. performed experiments; N.S., S.J., and L.G. analyzed data; N.S. prepared figures; N.S. and Y.P.J. drafted manuscript; M.B., R.C., and Y.P.J. interpreted results of experiments; R.C., M.K.R., and Y.P.J. approved final version of manuscript; M.K.R. and Y.P.J. conceived and designed research.

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