Original Article

Angiotensin II Potentiates Vascular Endothelial Growth Factor-Induced Proliferation and Network Formation of Endothelial Progenitor Cells

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Bone marrow-derived endothelial progenitor cells (EPCs) in the peripheral blood of adult animals and adult humans have been shown to play a role in neovascularization into neovascular structures. On the other hand, angiotensin II (Ang II) plays a role in the development of many vascular diseases. To investigate whether Ang II affects human vascular endothelial growth factor (VEGF)-induced EPCs proliferation and network formation. Reverse transcription-polymerase chain reaction analysis demonstrated that Ang II induced a significant increase of VEGF receptor kinase domain-containing receptor (KDR) mRNA in a dose- and time-dependent manner; the maximal increase, which was 3-fold the control value, occurred after a 4-h stimulation. In addition, flow cytometric analysis revealed that Ang II up-regulated KDR protein expression in human EPCs. Both the angiotensin type 1 (AT1) receptor antagonist (valsartan: 200 nmol/l) and the PKC inhibitor, bisindolylmaleimide (GFX: 10 μmol/l) reduced Ang II-induced KDR mRNA expression to almost the control level. The culture assay showed that Ang II dose-dependently enhanced VEGF-induced EPC proliferation by activating AT1 receptors, which was also confirmed by the colorimetric MTS assay with the electron coupling reagent mathosulfate. Finally, in a Matrigel assay, EPCs treated with both Ang II and VEGF were shown to be more likely to integrate into the network formation than those treated with VEGF alone. In conclusion, our data indicate that Ang II potentiates VEGF-induced human EPCs proliferation and network formation through the up-regulation of KDR. (Hypertens Res 2004; 27: 101–108)

Key Words: angiotensin II, vasculogenesis, endothelial progenitor cells

Introduction

Progenitor cells are primitive bone-marrow cells that have the capacity to proliferate, migrate, and differentiate into various mature cell types. Endothelial progenitor cells (EPCs) possess the ability to mature into the cells that line the lumen of blood vessels (1). Recent studies have provided increasing evidence that postnatal neovascularization does not consist exclusively of the sprouting of preexisting vessels, but also involves the contribution of bone marrow-derived EPCs (2, 3). EPCs have been isolated from peripheral blood, and have been shown to augment neovascularization of ischemic tissue (4). In light of these findings that EPCs contribute to postnatal neovascularization, an improved understanding of the regulation of EPCs could lead to new insights into the pathogenesis of vasculogenesis.

Angiotensin II (Ang II), the main effector of the renin-angiotensin system (RAS), may contribute to vessel growth regulation. Indeed, Ang II has been shown to regulate cell growth of vascular smooth muscle cells (VSMCs) (5, 6), and the induction of endothelin-1 in endothelial cells (7). Ang II also increases vessel density in the rat cremaster (8) and in the chorioallantoic membrane of the chick embryo (9), and

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activates in vivo angiogenesis in the rat subcutaneous sponge granuloma (10).

Although much effort has been focused on Ang II-induced angiogenesis, the effect of Ang II on the bone marrow-derived EPCs remains unresolved. Therefore, in the present study we examined the relation between EPC biological activity and Ang II. Our results showed that Ang II is a potent stimulator of vascular endothelial growth factor (VEGF)-induced proliferation and network formation of human EPCs through the induction of the VEGF receptor kinase domain-containing receptor (KDR).

Methods

Isolation of Mononuclear Cells and Cell Culture

EPCs were cultured according to a previously described technique (11). Briefly, peripheral blood mononuclear cells (PB-MNCs) were isolated from healthy volunteers by density gradient centrifugation with Histopaque 1077 (Sigma Chemical Co., St. Louis, USA). After purification with 3 washing steps, 10^6 PB-MNCs were plated on fibronectin-coated 6-well plates. Cells were cultured in endothelial basal medium-2 (EBM-2) (Clontech, Palo Alto, USA) supplemented with 3 µg/ml bovine brain extract, 30 µg/ml gentamycin, 50 µg/ml amphotericin B, 10 µg/ml human epidermal growth factor, and 5% fetal bovine serum (FBS). The cells were replated onto fresh medium on day 4 of culture. EPCs were harvested after 7 days of culture, replated in 4-well glass plates coated with fibronectin and gelatin, and cultured with EBM-2 supplemented with 0.5% bovine serum albumin (BSA) for 16 h. Then, EPCs were pretreated with Ang II (Sigma) at 1, 10, or 100 nmol/l or with vehicle for 12 h before the addition of 20 ng/ml recombinant VEGF (R&D Systems Inc., Minneapolis, USA). The cells were harvested 7 days after culture and re-seeded on a 96-well plate (1 × 10^4 cells) in 0.1 ml of EBM-2 medium supplemented with 0.5% BSA for 16 h. Then, the cells were pretreated with Ang II at 1, 10, or 100 nmol/l or with vehicle for 12 h before the addition of 20 ng/ml recombinant VEGF. After 24 h in culture, MTS/phenazine methosulfate (PMS) solution was added to each well and the cells were cultured for an additional 3 h, during which light absorbance at 490 nm was detected using an ELISA plate reader.

Mitogenic activity was assessed using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium assay (MTS) (CellTiter 96 AQ; Promega, Madison, USA). EPCs were harvested 7 days after culture and re-seeded on a 96-well plate (1 × 10^4 cells) in 0.1 ml of EBM-2 medium supplemented with 0.5% BSA for 16 h. Then, the cells were pretreated with Ang II at 1, 10, or 100 nmol/l or with vehicle for 12 h before the addition of 20 ng/ml recombinant VEGF. After 24 h in culture, MTS/phenazine methosulfate (PMS) solution was added to each well and the cells were cultured for an additional 3 h, during which light absorbance at 490 nm was detected using an ELISA plate reader.

Flow Cytometry

EPCs were harvested 7 days after culture, re-seeded on 6-well plates in EBM-2 medium supplemented with 0.5% BSA, and cultured for 16 h. They were then stimulated with 100 nmol/l Ang II or vehicle for 12 h. Fluorescence-activated cell sorter (FACS) analysis was performed on a FACS caliber flow cytometer (Becton Dickinson, Mountain View, USA). The cells were permeabilized with 9:1 methanol/PBS for 20 min at -20°C, then incubated for 30 min with mouse anti-human VEGF receptor-2/KDR (Sigma). Phycoerythrin (PE)-conjugated goat anti-mouse F(ab)2 (DAKO, Glostrup, Denmark) was used as a secondary antibody. All incubations were performed at 4°C following by fixation in 2% paraformaldehyde before measurement of fluorescence-activated cells using a flow cytometer.

Proliferative Activity Assay

To investigate the effect of Ang II on KDR mRNA expression, EPCs were treated with Ang II at 1, 10, or 100 nmol/l for the indicated times. To characterize the Ang II receptor subtype that is responsible for KDR mRNA induction, EPCs were pretreated with the angiotensin type 1 (AT1) receptor antagonist valsartan (200 nmol/l) for 15 min before Ang II stimulation. Previous studies have shown that protein kinase C (PKC) plays a role in Ang II-stimulated signaling pathways. Therefore, to determine the role of PKC in Ang II-induced KDR mRNA expression, EPCs were pretreated with a highly selective PKC inhibitor, bisindolylmaleimide (GFX: 10 µmol/l) for 15 min, followed by treatment with Ang II for 4 h. Total RNA was extracted by using an RNeasy RNA extraction kit (Qiagen, Chatsworth, USA). Briefly, cells were lysed in guanidinium isothiocyanate buffer, and RNA was purified following the manufacturer’s instructions. The purified RNA was suspended in diethyl pyrocarbonate (DEPC)-treated H2O. To generate cDNA, 1 µg total RNA was treated with DNaseI (Ambion, Austin, USA) to remove any contaminating genomic cDNA. The DNase-treated RNA (100 ng) was then converted into cDNA by using murine leukemia virus reverse transcriptase ( Gibco BRL Life Technologies, Bethesda, USA). The transcribed cDNA was then used for PCR amplification to estimate the expression of KDR. Two
specific primers matching the published sequences were used to identify and amplify KDR (sense primer, 5'-CTGGCATG GTCTTCTGTGAAGCA-3'; antisense primer, 5'-AATACC AGTGGATGTGATGCGG-3'). The PCR product was 790 bp in length. The amplification conditions were as follows: initial denaturation at 94ºC for 5 min, annealing at 60ºC for 1 min, and elongation at 72ºC for 30 s. The initial cycle was followed by 35 cycles of denaturation at 94ºC for 45 s, annealing at 60ºC for 45 s, and elongation at 72ºC for 2 min, followed by 7 min of extension at 72ºC. PCR products were then visualized on 1.5% ethidium bromide-stained agarose gels. β-Actin was amplified as a reference.

Matrigel Tubule Assay

EPCs were harvested 7 days after culture and re-seeded in 6-well plates on EBM-2 medium supplemented with 0.5% BSA, then cultured an additional 16 h. Then EPCs were pretreated with 100 nmol/l Ang II or vehicle for 12 h before culturing with 20 ng/ml recombinant VEGF for 24 h. Twenty-four well multidishes were coated with growth factor-re-
duced Matrigel (10 mg/ml; Becton Dickinson, Bedford, USA) according to the manufacturer’s instructions. For fluorescent labeling of EPCs, cells were incubated with dioctadecyl-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) at a concentration of 2.5 µg/ml PBS for 5 min at 37°C and 15 min at 4°C. DiI-labeled EPCs (1 × 10⁵) were incubated on the 24-well multidishes at 37°C for 24 h. Network formation images were observed using an inverted phase contrast microscope equipped with fluorescence digital imaging (Keyence, Osaka, Japan).

**Statistical Analysis**

Data are expressed as the mean ± SEM from at least 3 independent experiments. Statistical analysis was performed by 1-way ANOVA (LSD test) for multiple comparisons. Values of p<0.05 were considered to indicate statistical significance.

**Results**

**Ang II Stimulates VEGF Receptor 2 (KDR) mRNA and Protein Expression in Human EPCs**

To investigate the effect of Ang II on KDR mRNA expres-
PKC inhibitor, GFX (10 µmol/l), expression, human EPCs were treated with a highly selective inhibitor of PKC in Ang II-induced KDR mRNA expression is mediated through the AT1 receptor. Previous reports have shown that PKC plays a role in Ang II-stimulated signaling pathways (12). To determine the role of PKC in Ang II-stimulated KDR mRNA expression, human EPCs were treated with a highly selective PKC inhibitor, GFX (10 µmol/l), followed by treatment with Ang II. The pretreatment with GFX also reduced Ang II-induced KDR mRNA expression to nearly the control level.

Effect of Ang II on VEGF-Induced EPC Proliferation

The EPCs were characterized as adherent cells that were double-positive for both lectin and DiLDL uptake. As reported previously (11), when EPCs were incubated with VEGF, the number of adherent EPCs was increased compared with the control (Fig. 4). Next, we examined the effect of Ang II on VEGF-induced augmentation of EPC proliferation. As shown in Fig. 4B, Ang II enhanced VEGF-induced EPC proliferation in a dose-dependent manner, although Ang II itself had no effect on EPC proliferation. These results were also confirmed by the colorimetric MTS assay with the electron-coupling reagent methosulfate (Fig. 5). In addition, the MTS assay disclosed that the AT1 receptor antagonist valsartan significantly inhibited the effect of Ang II on VEGF-induced EPC proliferation (Fig. 5).

Ang II Enhances VEGF-Induced Network Formation in EPCs

A Matrigel tubule assay was performed to investigate the ability of EPCs to integrate into vascular structures. Previous reports have shown that Ang II potentiates VEGF-induced network formation in retinal microcapillary endothelial cells (13). First, we confirmed that Ang II enhanced VEGF-induced network formation in mature cells of another endothelial cell line, Human Umbilical Vascular Endothelial Cells (HUVECs) (Fig. 6A). Ang II alone had a modest effect on network formation of HUVECs. Next, we examined the effects of Ang II on VEGF-induced network formation in human EPCs. Because the lineage and exact phenotype of EPCs are not yet known, fluorescent tagging of EPCs with DiI was performed. As shown in Fig. 6B, fluorescent microscopy revealed that pretreatment of EPCs with Ang II followed by VEGF treatment potentiated network formation to a greater degree than treatment of EPCs with either VEGF alone or Ang II alone.

Discussion

Recent studies have demonstrated that atherosclerotic risk factors are inversely correlated with the number of EPCs (14, 15). Given the fact that EPCs contribute to postnatal neovascularization, an improved understanding of the regulation of EPCs could lead to new insights into the pathogenesis of vasculogenesis. Here we have shown that Ang II potentiates VEGF-induced proliferation and network formation of human EPCs through the up-regulation of VEGF receptor 2/KDR. In addition, we have shown that the up-regulation of KDR mRNA induced by Ang II can be abolished by either AT1 receptor antagonist or a PKC inhibitor.

VEGF exerts its biological effects through binding to two high affinity tyrosine kinase receptors, KDR/Flk-1 and Flt-1 (16, 17). Recent reports have shed some light on the role of Flt-1 and its effects on processes such as angiogenesis,
Fig. 6. Effect of VEGF-induced HUVECs and EPCs network formation. Both HUVECs and EPCs were stimulated with 20 ng/ml VEGF for 24 h in the presence or absence of pretreatment with Ang II for 12 h. Then, both HUVECs (A) and fluorescent-labeled EPCs (B) were seeded in growth factor-reduced Matrigel to induce network formation as described in the Methods. Representative images are shown.
hematopoiesis, and inflammation. Flt-1 has been shown to promote hematopoiesis by recruiting hematopoietic stem cells from the bone marrow, which favors differentiation and mobilization (18, 19). On the other hand, KDR/Flik-1 is implicated in the recruitment, differentiation, and proliferation of EPCs (20, 21). Therefore, we have focused on KDR/Flik-1 in the present study. VEGF, which binds to the KDR/Flik-1 receptor specifically, has been shown to strongly induce Akt phosphorylation in endothelial cells (22). Akt is a serine threonine protein kinase that is activated by a number of growth factors and cytokines in a phosphatidylinositol-3 kinase (PI3K)-dependent manner (23). Importantly, the PI3K/Akt pathway plays a significant role in mediating VEGF biological activity. Dinnmier et al. have shown that both VEGF and statins induce EPC differentiation via the PI3K/Akt pathway, as demonstrated by the inhibitory effect of pharmacological PI3K blockers or overexpression of a dominant negative Akt construct (11). We have previously demonstrated that oxidized LDL reduces the number of adherent EPCs in the presence of VEGF through the dephosphorylation of Akt (24). These findings led us to examine whether Ang II, which also plays an important role in vascular remodeling, regulates KDR/Flik-1 receptor as a means of potentiating vasculogenic effects in human EPCs. We performed RT-PCR analysis and found that Ang II stimulated KDR mRNA expression in human EPCs in a dose- and time-dependent manner. In addition, flow cytometric analysis revealed that Ang II up-regulated KDR protein expression in human EPCs.

Two major angiotensin receptor subtypes have been defined: AT\textsubscript{1} and AT\textsubscript{2} (25, 26). Most of the actions of angiotensin are mediated by the AT\textsubscript{1} receptor, whereas the actions of the AT\textsubscript{2} receptor are not well understood (27). The expression of AT\textsubscript{2} receptor is reported to be regulated by the developmental stage of tissues and is thought to be involved in tissue growth and differentiation (28, 29). The growth-promoting effect of Ang II in VSMCs has been reported to occur via the AT\textsubscript{1} receptor (30). In our experiments, both the up-regulation of KDR and the potentiation of VEGF mitogenic effects in Ang II-stimulated EPCs could be abolished by the pretreatment of the AT\textsubscript{1} specific receptor antagonist, suggesting that these effects occurred via the AT\textsubscript{1} receptor. However, our experiments did not clarify the role of the AT\textsubscript{2} receptor in the Ang II-induced effects on EPCs, and further studies will be needed on this subject. We made further analyses to delineate the signal transduction pathway responsible for the effect of Ang II on the increase of KDR mRNA. The AT\textsubscript{1} receptor is a G protein-coupled receptor and activates phospholipase C, which is known to induce the hydrolysis of phosphoinositol and the activation of PKC (27). Ang II-induced increases of several growth factors, such as platelet-derived growth factor A-chain (31), insulin-like growth factor I (32), and endothelin-1 (7), are reported to be mediated through the AT\textsubscript{1} receptor in conjunction with activation of PKC. The present study also suggested that KDR mRNA induction by Ang II is mediated predominantly in a PKC-dependent manner in human EPCs.

Current data suggest that EPCs play a significant role in neovascularization in ischemic tissue. In the Matrigel tube assay, we demonstrated that pretreatment with Ang II enhanced the VEGF-induced network formation of EPCs. Because this was a global assay evaluating multiple cellular processes involved in blood vessel growth, it is likely that Ang II enhances the VEGF-induced vascular activity in EPCs. Although Ang II alone had a modest effect on network formation of both HUVECs and EPCs, the underlying mechanism of its enhancement of VEGF-induced network formation remain unclear and warrant further study.

In conclusion, the results of the present study demonstrated that Ang II potentiates VEGF-induced proliferation and network formation of EPCs. The Ang II-induced enhancement of the biological activity of EPCs may affect the potentiation of vascular remodeling.

References


