Regulation of endothelial proliferation by the renin–angiotensin system in human umbilical vein endothelial cells

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Abstract

This study was performed in order to evaluate the role of angiotensin II in physiological angiogenesis. Human umbilical vein endothelial cells (HUVEC) were stained for angiotensin II type 1 receptor (AGTR1) immunocytochemically and for gene expression of renin–angiotensin system (RAS) components. The regulation of the angiogenesis-associated genes vascular endothelial growth factor (VEGF) and angiopoietins (ANGPT1 and ANGPT2) were studied using quantitative RT-PCR. Furthermore, we examined the effect of angiotensin II on the proliferation of HUVEC using Ki-67 as well as BrdU immunocytochemistry and investigated whether the administration of the AGTR1 blocker candesartan or the VEGF antagonist FLT1-Fc could suppress the observed angiotensin II-dependent proangiogenic effect. AGTR1 was expressed in HUVEC and the administration of angiotensin II significantly increased the gene expression of VEGF and decreased the gene expression of ANGPT1. Since the expression of ANGPT2 was not affected significantly the ratio of ANGPT1/ANGPT2 was decreased. In addition, a significantly increased endothelial cell proliferation was observed after stimulation with angiotensin II, which was suppressed by the simultaneous administration of candesartan or the VEGF antagonist FLT1-Fc. These results indicate the potential capacity of angiotensin II in influencing angiogenesis by the regulation of angiogenesis-associated genes via AGTR1. Since VEGF blockade opposed the effect of angiotensin II on cell proliferation, it is hypothesised that VEGF mediates the angiotensin II-dependent effect in concert with the changes in angiopoietin expression. This is the first report of the RAS on the regulation of angiogenesis-associated genes in physiology.


Introduction

Angiogenesis is a rare event in the healthy adult vasculature but is critical in pathological conditions such as solid tumours, chronic arthritis, collagen diseases, diabetic retinopathy and arteriosclerosis. Considerable advances have been made in controlling angiogenesis therapeutically, in order to treat these conditions, but further work is required to identify additional targets in the vascularisation process that may be inhibited to form the basis of safe and effective treatments, either as single agents or in combination with the inhibition of angiogenic factors like the vascular endothelial growth factor (VEGF).

The renin–angiotensin system (RAS) controls renal homeostasis and regulation of the vascular tone in the cardiovascular system and was recently suspected to be involved in the regulation of angiogenic processes. RAS components have been detected in endothelial and ovarian cells (Dzau 1988, Harata et al. 2006). Furthermore, angiotensin II has been described to influence reproductive processes such as ovulation (Mitsube et al. 2003, Ozcakir et al. 2004, Ferreira et al. 2007).

Angiotensin II is a multifunctional bioactive octapeptide converted from its precursors angiotensinogen and angiotensin I by the catalytic action of angiotensin I-converting enzyme (ACE). Angiotensin II mediates its effects by binding to two different receptors, angiotensin II type 1 receptor (AGTR1) and AGTR2 (Timmermans et al. 1992). The activating effects of angiotensin II are mediated via G-protein-coupled AGTR1 (De Paepe et al. 2001, Egami et al. 2003) and opposed via AGTR2 (Goto et al. 2002, Silvestre et al. 2002). In addition, angiotensin II has been proposed to participate in the regulation of the angiogenic processes (Suganuma et al. 2005) by influencing angiogenic factors such as VEGF and the angiopoietins in glomerular mesangial cells and senescent fibroblasts (Chen et al. 2005, Sanchez-Lopez et al. 2005, Wang et al. 2006).

VEGF is a principal promoter of angiogenic process, which acts by inducing a series of events, including differentiation of endothelial cells, tube formation and vascular maturation (Flamme et al. 1997, Risau 1997). Targeting VEGF by the application of VEGF antibodies or VEGF antagonists has become an approach in anti-angiogenic treatment (Wulff et al. 2001a, 2002a,
Bernard-Marti et al. 2006). A further approach might be to target up- or downstream effectors of the cellular signalling, which has not yet been elucidated fully. While VEGF is the prime initiator of angiogenesis, the formation of a mature vascular network requires the coordinated action of many factors. These include the angiopoietins, which act via the tyrosine kinase receptor TIE2 (Maisonpierre et al. 1997). ANGPT1 enhances the maturation and stability of newly formed blood vessels, by recruiting perivascular support cells such as pericytes (Suri et al. 1998, Papapetropoulos et al. 1999), whereas ANGPT2 can act antagonistically blocking ANGPT1-mediated receptor phosphorylation (Fraser 2006). Therefore, VEGF mediates vasculogenesis, whereas the angiopoietins are more involved in the process of vascular remodelling during angiogenesis (Maisonpierre et al. 1997, Wulff et al. 2002b).

The question of the potential interrelation of angiotensin II with VEGF and the angiopoietins in endothelial cells and its functional role concerning the regulation of angiogenesis in physiology has not yet been established.

To begin to elucidate the potential regulatory functions of angiotensin II in physiology, we investigated the angiotensin II-induced effects on endothelial cells in vitro. The expression of angiogenesis-related genes VEGF and ANGPT1 and ANGPT2 in human umbilical vein endothelial cells (HUVEC) was examined after the administration of angiotensin II. In addition, we investigated the proliferation rate of HUVEC in vitro after stimulation with angiotensin II and assessed whether the AGTR1 blocker or the VEGF antagonist FLT1-Fc could suppress the angiotensin II-dependent increased proliferation rate of HUVEC in order to evaluate the potential of these inhibitors in the regulation of angiogenesis.

Results

We detected the AGTR1 protein by immunohistochemistry in the plasma membrane of HUVEC (Fig. 1). Furthermore, we showed the gene expression of RAS components angiotensinogen, ACE, AGTR1 and AGTR2 (Fig. 2), and the gene expression of the angiogenesis-associated genes VEGF, ANGPT1 and ANGPT2 in HUVEC.

The stimulation of HUVEC with angiotensin II (Fig. 3) was followed by a significantly increased expression of VEGF (in controls, 0.12733 ± 0.005 1/Δcts versus after stimulation with angiotensin II, 0.14633 ± 0.0103 1/Δcts; P < 0.05) and the decreased expression of angiopoietin 1 (in controls, 0.057 ± 0.001 1/Δcts versus after stimulation with angiotensin II, 0.0533 ± 0.00088 1/Δcts; P < 0.05). However, the expression of angiopoietin 2 was not influenced significantly by angiotensin II (in controls, 0.0707 ± 0.00186 1/Δcts versus after stimulation with angiotensin II, 0.0733 ± 0.00186 1/Δcts; P = 0.144).

In order to investigate the proposed proangiogenic effect of angiotensin II on HUVEC, we measured the proliferation of these cells (Fig. 4a–f) using quantitative Ki-67 immunocytochemistry as well as the BrdU assay.

Figure 1 Angiotensin II type 1 receptor (AGTR1) in HUVEC cells is localised to the vicinity of the plasma membrane (arrows).

A significant increase in the proliferation after stimulation of HUVEC with angiotensin II was detected (Figs 4a–b and 5), which was abolished by the simultaneous administration of the VEGF inhibitor FLT1-Fc (Figs 4c and 5). A comparative but even higher downregulation was observed after simultaneous stimulation of HUVEC with angiotensin II and the AGTR1 blocker candesartan (Figs 4d and 5). While the administration of FLT1-Fc alone (Fig. 4e) revealed a comparative proliferation to controls, the treatment of HUVEC with candesartan alone (Fig. 4f) significantly suppressed the normal endothelial proliferation rate.

Discussion

The present study was performed in order to evaluate the role of angiotensin II as a potential regulator of physiological angiogenesis. Here, we show for the first time that angiotensin II induces endothelial proliferation by...
increasing the expression of the principal angiogenic factor VEGF, and influencing the ratio of ANGPT1/ANGPT2. Moreover, it is demonstrated that the increased endothelial proliferation was significantly suppressed by administration of the AGTR1 blocker candesartan or the VEGF antagonist FLT1-Fc. These data provide the first evidence that angiotensin II is involved in the regulation of physiological angiogenesis via binding to the AGTR1, which affects the main angiogenic factors VEGF and the angiopoietins.

VEGF is considered to be the most important specific stimulator of endothelial cell proliferation, acting through two tyrosine kinase receptors VEGFR1 (FLT1) and VEGFR2 (KDR) (Ferrara & Davis-Smyth 1997), and its critical role in the regulation of physiological angiogenesis has been demonstrated in the non-human primate reproductive tract (Wulff et al. 2000, 2001a, 2001b, 2002a, Fraser et al. 2006). Using specific VEGF antagonists to neutralise VEGF effects, it has been demonstrated that ovarian angiogenesis and follicular and corpus luteum development are severely compromised. Little is known about the role of other potential regulatory factors such as angiotensin II. The angiotensin II system has been localised in the human corpus luteum (Harata et al. 2006) showing the expression of angiotensin II and AGTR1. We detected the components of the RAS in HUVEC, indicating that this system could be involved in the autocrine or paracrine regulatory processes of endothelial proliferation. Although it has to be taken into account that HUVEC are foetal cells, this finding is underlined by our observation that angiotensin II

![Figure 3](image-url) Real-time RT-PCR quantification (1/acts) of VEGF (a), angiopoietin 1 (b) and angiopoietin 2 (c). Expression is normalised to 18S RNA as internal standard in HUVEC cells. Control (C); stimulation with angiotensin II (A). *Indicates significant differences (P<0.05).

![Figure 4](image-url) Ki-67-positive HUVEC: control (a), incubation with angiotensin II (b), angiotensin II and FLT1-Fc (c), angiotensin II and candesartan (d), FLT1-Fc (e) and candesartan (f). The left picture shows Ki-67-positive cells, the right one represents all cell nuclei.
induces VEGF gene expression as well as endothelial cell proliferation, thus angiogenesis. The administration of the VEGF antagonist FLT1-Fc significantly inhibited the observed angiotensin II-dependent increased proliferation of cells, proving that VEGF is a downstream angiogenic mediator of RAS.

The organisation of blood vessel formation and regression also involves additional factors. These include ANGPT1 and ANGPT2 (Maisonpierre et al. 1997, Wulff et al. 2000), which are of particular interest because both act via the same TIE2 receptor. ANGPT1 stimulates the TIE2 receptor inducing pericyte recruitment leading to the stabilisation of the vessel. The binding of ANGPT2 is not followed by receptor activation, so that potentially the vessel becomes destabilised, a scenario that may facilitate the action of the proangiogenic factor VEGF so that endothelial proliferation can be induced. The local balance of ANGPT1/ANGPT2 determines whether vessel stabilisation or angiogenesis occurs in the presence of VEGF. Our results showed that angiotensin II suppresses ANGPT1 gene expression, whereas ANGPT2 was not affected. This results in a decreased ANGPT1/ANGPT2 ratio causing a predominant ANGPT2 effect, so that endothelial proliferation may be induced by VEGF. These results indicate that the angiopoietins may be a second downstream mediator of the RAS and lead to the hypothesis that the RAS influences several downstream mediators of angiogenesis. This is further underlined by our finding that the inhibition of the AGTR1 has a more pronounced effect of suppression of endothelial cell proliferation than the inhibition of VEGF alone. It may well be that there are additional downstream effectors other than VEGF and angiopoietins, which make the idea of RAS-dependent control an intriguing possibility for the regulation of angiogenesis.

In a variety of animal models, it has been shown that ACE inhibitors have an anti-angiogenic activity (Volpert et al. 1996, Hii et al. 1998, Yoshiji et al. 2001, 2002, Lindberg et al. 2004). Since angiotensin II is produced not only by ACE but also by other enzymes (Muramatsu et al. 2000), the use of ACE inhibitors alone is likely to be insufficient to block angiotensin II-mediated effects on cells. We have demonstrated that the stimulating effects of angiotensin II on the endothelium are mediated by binding to AGTR1. Therefore, we consider that selective inhibition of AGTR1 might be more efficient than ACE inhibition. A further argument for selective inhibition of the AGTR1 may be the fact that autocrine mechanisms within the endothelial cells may be inhibited. We observed decreased proliferation rates in HUVEC after the administration of candesartan alone without the stimulation of angiotensin II compared with the endothelial cell controls. The observed candesartan effect might be explained by the inhibition of intrinsically synthesised angiotensin.

In summary, we have shown that angiotensin II can participate in the regulation of angiogenesis in endothelial cells in vitro via influencing different angiogenesis regulatory genes. In addition, the inhibition of AGTR1 was more effective than the administration of FLT1-Fc in inhibiting angiogenesis, which leads us to propose that an AGTR1 blocker might be useful as an anti-angiogenic agent, opening the intriguing possibility of developing a novel targeted therapy for interfering with angiogenesis.

Materials and Methods

HUVEC isolation from umbilical cords

HUVEC were isolated from multiple segments of normal term umbilical cords, pooled and cultured in the endothelial cell growth medium (Promocell GmbH, Heidelberg, Germany).

Cell cultures for HUVEC

HUVEC were cultivated in BD Falcon primaria flasks with the endothelial cell growth medium (Promocell GmbH), 10% foetal calf serum (PAA Laboratories, Pasching, Austria), penicillin-streptomycin and supplement mix (Promocell GmbH). The cells were incubated at 37 °C under saturated humidity and an atmosphere containing 5% CO₂. Media were changed every 48 h. The cells were stimulated with 9.6×10⁻⁸ mol/l, 9.6×10⁻⁷ mol/l (gene expression) and 9.6×10⁻⁶ mol/l (proliferation assay) of angiotensin II (Sigma–Aldrich) as well as simultaneous stimulation with the same concentrations of angiotensin II and 1 μg/ml candesartan (Astra Zeneca). The cells were lysed after 48 h and RNA was isolated.

Immunofluorescence cytochemistry

Cells were cultured on glass coverslips, rinsed with PBS and fixed in 100% methanol for 10 min on ice. The cells were washed in

![Figure 5](image-url)
PBS for 5 min and the proteins blocked with BSA (Sigma) for 15 min at room temperature. The cells were incubated overnight with rabbit polyclonal anti-AGTR1 (1:50) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4 °C. The cells were then rinsed and incubated with FITC-labelled goat anti-rabbit IgG (Zymed, South San Francisco, CA, USA) for 30 min at room temperature. After three washing steps, the coverslips were mounted on glass slides using Vectashield and viewed using Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan).

For Ki-67 detection, HUVEC were cultivated on chamber slides as described above and incubated with angiotensin II (9.6 × 10^{-6} mol/l), angiotensin II (9.6 × 10^{-6} mol/l) and FLT1-Fc (50 µg/ml), angiotensin II (9.6 × 10^{-6} mol/l) and candesartan (1 µg/ml), FLT1-Fc (50 µg/ml) (Sigma–Aldrich) and candesartan (1 µg/ml) for 48 h. The cells were then rinsed with PBS and fixed in 100% methanol for 10 min on ice. The cells were washed in PBS for 5 min and the proteins were blocked with BSA (Sigma–Aldrich) for 10 min at room temperature. The cells were incubated overnight at 4 °C with anti-Ki-67 (1:100) (Novocastra, Newcastle upon Tyne, UK). They were then rinsed with PBS and incubated with goat anti-rabbit IgG (1:100; Zymed) for 30 min at room temperature. After three washing steps with PBS the slides were mounted using Vectashield with DAPI and viewed using an Olympus BX51 fluorescence microscope (Olympus).

For BrdU detection, the cells were treated with angiotensin II (9.6 × 10^{-6} mol/l), angiotensin II (9.6 × 10^{-6} mol/l) and FLT1-Fc (50 µg/ml), angiotensin II (9.6 × 10^{-6} mol/l) and candesartan (1 µg/ml), FLT1-Fc (50 µg/ml) (Sigma–Aldrich) and candesartan (1 µg/ml) for 24 h and the incorporation of BrdU was detected using BrdU Labelling and Detection Kit 1 (Roche) according to the manufacturer’s instructions.

### RNA isolation

Total RNA from HUVEC was extracted using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. The RNA product was quantified by absorbance at 260 nm. Total RNA (2.5 µg) was reverse transcribed into cDNA using random (N6) primers according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA).

### RT-PCR analysis

PCR amplification was carried out with 0.2 mM dNTP, 10 µM forward primer, 10 µM reverse primer, 1.5 mM MgCl2 and 1 U Taq polymerase (Qiagen) in the recommended buffer. Two microlitres of a 1:10 diluted transcribed cDNA were used as the template. The cycling conditions were 45 cycles in an Eppendorf Thermocycler at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s after the initial denaturation step (94 °C for 5 min). A 7 min final extension step at 72 °C was added. The total volume of the PCR was 25 µl. The primers were designed according to the known sequences of VEGF in order to amplify a DNA fragment of 367 bp (forward primer: 5'-CGG GCC TCC GAA ACC ATG AAC TTT-3'; reverse primer: 5'-CTG GTT GGT CCC AGT GAG GAT GTT CTT-3'), of angiotensin II type 1 receptor (AGTR1) in order to amplify a DNA fragment of 330 bp (forward primer: 5'-GGA AAC ACG TTG GTG ATG AT-3'; reverse primer: 5'-GCA GCC AAA TGA TGC AG-3'), angiotensin II type 2 receptor (AGTR2) in order to amplify a DNA fragment of 263 bp (forward primer: 5'-CTG CTG TTG AGT CTT CTT-3'; reverse primer: 5'-ACT CTC TCT TTT CCC TCC TGG CAG CC-3'), of angiotensinogen 1 in order to amplify a DNA of 265 bp (forward primer: 5'-TTT AGA ACC ACA CGG CTA CCA T-3'; reverse primer: 5'-TCT TCC TGT TGT TTT CCT TCC ATT-3'), of angiopoietin 2 in order to amplify a DNA of 172 bp (forward primer: 5'-TAA GCA GGC CAT TGC TGC TAA AGA-3'; reverse primer: 5'-CAT ATG GTC AAT TCT CAG CCC GCC TCC TC-3') and GAPDH in order to amplify a DNA of 657 bp (forward primer: 5'-CTG GGC CTT AGT ACG TCG-3'; reverse primer: 5'-TTG ACA AAG TGG TCG TGC A-3'). To verify the specificity of the PCR, the PCR products were sequenced, revealing a sequence of the gene of interest in all cases. Bands were visualised after electrophoresis on a 2% agarose gel (Invitrogen) by staining with ethidium bromide. The pictures were taken with a camera system of Alpha Corporation (San Leandro, CA, USA).

### TaqMan analysis

Oligonucleotide primers (MWG Biotech, Ebersberg, Germany) were designed using the Primer Express 1.0 software. The probes consisted of an oligonucleotide, labelled at the 5'-end with the reporter dye 5-carboxyfluorescein (FAM) and the 3'-end with the quencher N,N',N'-tetramethyl-6-carboxyrhodamine. PCR was carried out using a TaqManTM Gold kit, as recommended by the manufacturer. The PCR mixture contains 100 nM of the probe and 2 µl of a 1:10 diluted reverse-transcribed RNA as template. The amplification and detection of specific products was performed using the ABI Prism 7700 sequence detection system (PE Applied Biosystems). The quantity of cDNA for VEGF and angiopoietin 1 and 2 was normalised to the quantity of 18S RNA in each sample by dividing the fluorescence values of the investigated genes amplification through the fluorescence values of 18S RNA amplification. Since these delta ct values are negatively correlated with the amount of gene expression, they were converted to 1/delta ct in order to avoid confusion.

For quantification of VEGF and angiopoietin 1 and 2 expression, the corresponding kits from Applied Biosystems were used performing under conditions according to the instructions of the manufacturer.

### Statistical analysis

Statistical analysis for Taq-Man experiments were performed using SPSS for Windows (SPSS Inc., Chicago, IL USA). Data obtained from three individual experiments with different treatments compared with controls were tested for significant differences using a two-tailed, unpaired t-test. The differences were considered to be significant at P<0.05. Data were given as
mean ± s.e.m. Statistical analyses for the proliferation assay were tested using ANOVA, followed by Duncan’s multiple range test.

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