Antagonism of the Renin-Angiotensin System Can Counteract Cardiac Angiogenic Vascular Endothelial Growth Factor Gene Therapy and Myocardial Angiogenesis in the Normal Heart


Background: The aim of this study was to test the hypothesis that angiotensin converting enzyme inhibition or angiotensin II antagonism can counteract cardiac human vascular endothelial growth factor-A165 (phVEGF-A165) induced angiogenesis.

Methods: Mice were given a single intramyocardial injection of phVEGF-A165. Either enalapril or candesartan was given subcutaneously for 10 consecutive days. Hearts were harvested and capillary count was performed by immunohistochemistry. With similar design, groups of mice were sacrificed after 24 h for the determination of tissue expression of phVEGF-A protein, mRNA expression of mouse VEGF-A, and VEGF receptors 1 and 2, after pEGFP-Luc transfection for luciferase expression.

Results: Increased myocardial capillary density (P < .02) induced by phVEGF-A165 was counteracted by both enalapril (P < .07) and candesartan (P < .02) and then did not differ from control values. We found that phVEGF-A165 induced myocardial hVEGF-A expression (110 ± 15 pg/heart, P < .0001). Both enalapril and candesartan decreased (P < .01) expression of hVEGF-A to a level not different from control values. Although phVEGF-A165 up-regulated (P < .0001) mVEGFR-2, addition of candesartan downregulated endogenous mVEGF-A (P < .0001) and mVEGFR-2 (P < .0001) below the level in normal myocardium. Enalapril or candesartan did not effect luciferase expression.

Conclusions: Enalapril and candesartan both specifically inhibit phVEGF-A165 induced myocardial angiogenesis in the normal heart. The mechanism of inhibition is a combination of inhibition of cardiac hVEGF-A expression and of decreased endogenous expression of the mVEGF ligand and receptor system. Am J Hypertens 2005;18: 1347–1352 © 2005 American Journal of Hypertension, Ltd.

Key Words: Angiogenesis, gene therapy, angiotensin converting enzyme inhibitor, angiotensin II antagonist, vascular endothelial growth factor.

The renin-angiotensin system (RAS) is essential for regulation of blood pressure and homeostasis. Inhibition of the RAS by angiotensin-converting enzyme inhibitor (ACEi) and angiotensin II type 1 receptor (AT1) antagonism has cardio protective roles after myocardial infarction in experimental1 and clinical2 studies. In the compromised myocardium in a rat myocardial infarction model3,4 or in obese Zucker rats5 RAS inhibition improved myocardial angiogenesis in parallel with decreased left ventricular filling pressure. However evidence suggests that RAS is involved both in vascular endothelial growth factor (VEGF) upregulation and angiogenesis6–8 and in counteracting angiogenesis.9–11 Angiotensin II, the active component of the RAS, mediates most of the angiogenic stimulus in both endothelial and smooth muscle cells,12 possibly through the activation of VEGF.13 The substance VEGF is an important endothelial cell specific growth factor that has potent angiogenic effect mediated by its receptor VEGFR-2.14 An angiogenic gene therapy phase II clinical trial with VEGF-A in severe ischemic heart disease has shown promising efficacy.15,16 Both ACEi and angiotensin II antagonists are commonly used in...
patients with ischemic heart disease, who are potential candidates for angiogenic gene therapy. Enhanced angiotensin II activation is detected during myocardial ischemia\textsuperscript{17} which may also upregulate endogenous angiogenesis a a natural process. However, also enhanced myocardial capillary growth is suggested as a direct consequence of hypoxia and myocardial stretch\textsuperscript{18} may underscore complex molecular mechanisms of RAS inhibition involving myocardial angiogenesis. The aim of this study was to test the hypothesis that ACEi or angiotensin II antagonism could counteract angiogenesis induced by myocardial overexpression of phVEGF-A\textsubscript{165}.

Material and Methods

The phVEGF-A\textsubscript{165} was prepared as previously described.\textsuperscript{19} Plasmid pEGFP-Luc (CloneTech, USA) is a reporter plasmid driven by a cytomegalovirus promoter-enhancer. The plasmid expresses green fluorescent protein (GFP) and luciferase after transfection. Placebo plasmid contains the phVEGF-A\textsubscript{165} backbone but without VEGF-A cDNA. In this study we used enalaprilat (Renitec 1 mg/mL, MSD, Sweden) and candesartan cilexetil (Atacand 4 mg, [AstraZeneca, Sodertalje, Sweden] pulverized and mixed with 4 mL of saline to get a concentration of 1 mg/mL).

Intramyocardial Cardiac Gene Transfer

The local animal ethics committee approved the study. Male C57BL/6 mice (B&K, Sweden), 6 to 8 weeks of age and weighing 20 to 25 g, were used. Mice were anesthetized with a 100-μL intraperitoneal injection consisting of combination of midazolam (5 mg/kg) and medetomidin (0.5 mg/kg) diluted with normal saline, then intubated and adapted to a ventilator (Zoovent, UK). A left thoracotomy was performed, and the heart was visualized by retracting the pericardial sac. The left ventricular wall was targeted for direct intramyocardial injection of phVEGF-A\textsubscript{165} (5 μg in 10 μL normal saline)\textsuperscript{20–22} or placebo plasmid or saline alone with an insulin syringe. Aspiration was done to confirm that the injection was not made into the ventricular cavity. After the wound was closed in layers, the mouse was given a combination of atipamamezol (2.5 mg/kg) and flumazenil (0.5 mg/kg) for termination of anesthesia and was kept under a heating lamp until awake.

With the above protocol each mouse was given a single intramyocardial injection of phVEGF-A\textsubscript{165} (5 μg in 10 μL normal saline). From day 1, after recovering from the operation, one group (n = 7) of mice received an subcutaneous injection of enalapril (30 mg/kg) daily for 10 consecutive days, and a second group (n = 7) received candesartan (20 mg/kg). Three separate groups of mice (n = 7) received intramyocardial injection of normal saline (10 μL) or placebo plasmid or phVEGF-A\textsubscript{165} as controls. On day 10 whole ventricles were harvested for cryosection and capillary count performed by histochemistry. Human VEGF protein expression was determined in subsets (n = 5/group in each of 3 groups) of mice after 24 h of phVEGF-A gene transfer and also with candesartan and enalapril treatment. Two additional groups of mice (n = 5/group) were injected phVEGF-A\textsubscript{165}, one group received candesartan treatment (20 mg/kg) and the other as saline injected control. After 24 hours mouse endogenous VEGF system at mRNA level was determined from left ventricular tissue (10 mg).

Another three groups of mice were injected with plasmid pEGFP-Luc (5 μgm in 10 μL saline) into the myocardium. One group was as control (n = 6) and the other two (n = 7/group) were treated with either enalapril or candesartan in the above specified doses. The ventricles from these three groups were harvested after 24 h for luciferase expression.

Protein Expression of phVEGF-A

The hVEGF-A protein was determined from the heart 24 h after injection. Briefly, immediately after euthanasia the hearts were taken out individually, cut into small pieces, and stored in liquid nitrogen. Excess blood was rinsed off with 20 mmol/L HEPES, pH 7.4, buffer. Thereafter heart tissue was taken into an Eppendorf tube with 400 μL homogenization buffer (20 mmol/L HEPES pH 7.4, 1.5 mmol/L ethylenediaminetraacetate, 0.5 mmol/L phenyl-methylsulfonyl fluoride, 0.5 mmol/L benzamidine, and 10 μg/mL trypsin inhibitor) and minced with a homogenizer knife for 1 min. Each homogenized substance was taken to another Eppendorf tube and centrifuged for 10 min at 14,000 rpm (4°C). Finally the supernatant was collected into two new Eppendorf tubes and frozen at −70°C. Later all frozen samples were analyzed at the same assay for hVEGF-A protein with a standard human VEGF immunoassay detection kit (Quankine, R&D Systems). The coefficient of variation (CV%) for a single determination was 6%.

Protein Expression of Luciferase

After 24 h the hearts were individually harvested in liquid nitrogen for luciferase expression. Hearts were homogenized on ice with 200 μL freshly prepared luciferase lysis buffer. The homogenate substance was centrifuged for 10 min at 14,000 rpm at 4°C. Luciferase activity in the hearts was determined according to the manufacturer’s instructions (Promega, Madison, WI).

Lectin Histochemistry for Capillary Morphology and Density

After 10 days the mice were sacrificed and hearts were immediately collected. The ventricles was dissected into two parts, embedded in Oct compound (Histolab), frozen in liquid nitrogen, then kept at −70°C until sectioned. Sections (5 μm) were fixed in ice-cold acetone for 10 min. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide and then incubated with Griffonia Bandeiraea Simplicifolia Isolentin B4 (GSL-I-B4, Vector Laboratories) followed by a second incubation...
with streptavidin-biotin-horseradish peroxidase complex (ABComplex, Dako). Finally capillaries were visualized by 3,3-diaminobezedine (DAB) with supplementation of 3% hydrogen peroxide. Capillaries were counted at (40× magnification) from photographs taken with a liquid crystal display camera (Olympus, Tokyo, Japan) connected with a microscope. From each mouse heart photos around the injection scar in the left ventricular wall from adjacent to three to four sections (mean number of photographs taken, seven) were counted for capillary analysis. Capillary count was analyzed with an image analysis program according to pre-set criteria regarding the size and developed color (Micro Image, Olympus, Tokyo, Japan). The quality of computer analysis had previously been checked against manual counting. The automated count was checked by a blinded count.

**RNA Isolation and cDNA Synthesis**

The RNA was extracted from fresh-frozen ventricular tissue, and cDNA synthesis was performed by random hexamer primers, as described before.22

**Statistical Analysis**

Results are expressed as the mean ± standard error of the mean. The Student unpaired t test was used to reject the null hypothesis. A P value < .05 was considered to be significant. Coefficients of variation (CV%) for a single determination were calculated as described.23

**Real-Time Polymerase Chain Reaction**

Real-time polymerase chain reaction was used for measurement of specific mRNAs (ABI-PRISMA 7700 Sequence Detector), which was also described earlier.22 The oligonucleotide sequences for the primer pairs and probes used were as follows: VEGF: 5′ CCACGTCAGAGAGCAACATCA, 3′ TCATTCTCTAGCCGCTTTG; Probe: FAM-CAGGGGAAAATACAGGA-TAMRA; VEGFR-1: 5′ TGTGAAACGGCTGCCCTATGATG, 3′ GCCAAATGCAGGCCTTGAA; Probe: FAM-CCCAGGATTGGAACC-TAMRA; VEGFR-2: 5′ TCTGGCTCCTTCTTGTCATTGTC, 3′ GCTCATCCAGGGCAATTCAT; Probe: FAM-A CGGGCATCAGGA-TAMRA, 18 S supplied as a TaqMan Reagents kit (Applied Biosystems) with TAMRA quencher and used according to the manufacturer’s instructions. Target gene and 18S were amplified in a single-plex experiment in duplicate samples.
Results

Myocardial Gene Transfer

Hematoxylin and eosin staining were used to show scar formation at the injection site (Fig. 1A) in the left ventricular wall, which validates successful myocardial gene transfer. In addition, 5-μm ventricular sections were analyzed to detect GFP transfected cardiomyocytes (Fig. 1B) that were also found close to the injection scar.

Expression of hVEGF-A and Luciferase

Analysis of phVEGF-A165 expression analysis at different time points in groups of mice showed maximal levels between 24 and 48 h (Fig. 2). After 24 h, the hearts injected with phVEGF-A165 alone showed expression of hVEGF-A (106 ± 27 pg/mL), whereas hVEGF-A was not detected in hearts treated either with saline or placebo plasmid injection. Compared with treatment with phVEGF-A165 alone, addition of candesartan or enalaprilat treatment resulted in markedly lower myocardial hVEGF-A expression (P < .02 and P < .05, respectively). There was no difference between candesartan and enalaprilat treated groups (Fig. 3).

After pEGFP-Luc transfection, relative luciferase units were 276 ± 69 after luciferase alone, 152 ± 39 after enalapril, and 197 ± 62 after candesartan. Analysis of variance showed no statistical difference among the three groups.

Expression in mRNA of mVEGF-A, mVEGFR-1, and mVEGFR-2 24 h after sham operated control or myocardial injection of phVEGF-A165 with or without intraperitoneal administration of candesartan 20 mg/kg daily. Values are given as mean with SEM. Analysis of variance showed statistical inhomogeneity for all three specimens. P values are for group differences versus baseline values.

FIG. 5. Capillary density after 10 days from a single intramyocardial injection of either placebo plasmid or phVEGF-A165 with or without enalapril or candesartan (n = 7/group). Values are mean ± SEM. Abbreviations as in Fig. 3.

FIG. 6. Representative figures of lectin stained myocardium 10 days after injection 100 to 200 μm from injection scar. Arrows indicate thin-layered and sprouting capillary structures.
Endogenous VEGF System in Mice

Myocardial phVEGF-A165 gene transfer did not affect mVEGF-A and mVEGFR-1 expression in the myocardium, whereas mVEGFR-2 was doubled \((P \leq .0001)\) compared with values in control hearts (Fig. 4). Administration of phVEGF-A165 combined with candesartan decreased expression of mVEGF-A \((P \leq .04)\), mVEGFR-1 \((P \leq .02)\), and mVEGFR-2 \((P \leq .0001)\) compared with values in saline treated controls.

Capillary Analysis

After 10 days of phVEGF-A165 gene transfer myocardial capillary density was increased by 16% compared with control \((P \leq .02)\) (Fig. 5). Two groups of control saline or placebo plasmid induced capillary densities \((2999 \pm 113 \text{ and } 2917 \pm 68 \text{ cap/mm}^2 \text{ respectively})\) were not different from values in non-injected myocardium \((2955 \pm 51 \text{ cap/mm}^2)\). In addition to a phVEGF-A165 induced increase in density of normal capillaries, thin layered and sprouting capillary structures were observed, which were closely located to the injection site in the left ventricular myocardium (Fig. 6). Such capillary structures were not observed in the placebo plasmid group and not seen often in mice treated with candesartan or enalapril (Fig. 6). No vascular tumor structures were observed.

Mouse hearts injected with phVEGF-A165 and subsequent treatment with enalapril or candesartan showed a decrease in capillary density \((P < .07 \text{ and } P < .02, \text{ respectively})\) compared with treatment with VEGF alone (Fig. 5). The capillary density levels were not statistically different from those in the placebo group.

Discussion

In this study, we found that transient overexpression of VEGF-A165 induced angiogenesis in the normal mouse myocardium, and that RAS inhibition counteracted this angiogenic stimulus. Mechanistically this RAS inhibition effect was associated with inhibition of VEGFR-2 expression and with decreased myocardial hVEGF-A content. No interference occurred with luciferase expression, suggesting that RAS inhibition does not interfere with cellular and nuclear uptake of the plasmid or with the efficacy of the viral CMV promoter. The effect of the RAS inhibition thus is not caused by a general inhibition of protein synthesis but may be caused by specific inhibition of VEGF-A protein expression. The decreased myocardial hVEGF-A protein content therefore could be caused by a RAS inhibition induced decreased transcription or increased elimination. As RAS inhibition has been shown to counteract VEGF expression and angiogenesis in several species, \(^9,24–27\) the effect is not species dependent.

Endothelial cells are an important source of angiotensin II and ACE. \(^28\) Proangiogenic function of angiotensin II is mediated by activation of AT1 receptor, which in turn stimulates expression of both VEGF \(^13\) and its receptors. \(^29\)

The VEGF ligand/coceptor system plays a critical role in angiogenesis, particularly through the activation of VEGFR-2 receptor. \(^18\) The substance ACEi causes increased levels of kinins, which can stimulate endothelial nitric oxide (NO) and prostaglandin production, thereby effecting vasodilation, and may also act via NO as an angiogenic stimulus. Antagonism of AT1 receptor causes additional increments in kinins as well as NO levels, because angiotensin II can stimulate NADH oxidase in the vascular wall, generating superoxide anions and causing degradation of NO. \(^30\) In addition angiotensin II production could be an independent pathway to ACE-like activity by stimulation of chymase formation, contributing to incomplete blockade of the RAS by ACEi. \(^31\) Interestingly, the balance between AT1 and AT2 receptor expression might be one factor involved in these opposing effects of RAS activity and inhibition. Stimulation of AT1 receptors promote angiogenesis and vasoconstriction, whereas AT2 receptor stimulation counteracts angiogenesis and vasodilation. \(^32\) Myocardial infarction in AT1 deficient mice also showed low myocardial infiltration of cells responsible for local cytokine regulation and resulted in reduced neovascular growth, suggesting a strong proangiogenic activity of AT1 receptor during ischemia. \(^33\)

In contrast, ACEi was reported to increase capillary density in skeletal muscle in hind limb ischemia. \(^6–8\) Inhibition of the RAS in compromised myocardium in a rat myocardial infarction model \(^33,4\) or in obese Zucker rats \(^5\) improved myocardial angiogenesis in parallel with decreased left ventricular filling pressure. These results emphasize the complexity of RAS actions as well as diverse actions on different tissue systems. It is evident that in pathologic conditions involving vasoconstriction and in the compromised heart, increased shear stress and lowering of blood pressure has a secondary effect that overrides the direct antiangiogenic effect by RAS inhibition reported by us and others. \(^10,11,27\)

In conclusion, in the noncompromised heart, enalapril and candesartan were found to inhibit phVEGF-A165 induced cardiac angiogenesis. Mechanistically this inhibition relates to downregulation of hVEGF expression and to simultaneous downregulation of the mouse endogenous VEGF system.

References


