Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular and Cellular Cardiology 35 (2003) 277–286
Journal of Molecular and Cellular Cardiology
www.elsevier.com/locate/yjmcc

Original Article

Cardiac myofibroblasts: a novel source of vascular endothelial growth factor (VEGF) and its receptors Flt-1 and KDR

Vishnu Chintalgattu, Devi M. Nair, Laxmansa C. Katwa *

Department of Physiology, Brody School of Medicine, East Carolina University, 600 Moye Building, Greenville, NC 27858, USA

Accepted 17 December 2002

Abstract

Vascular endothelial growth factor (VEGF), produced predominantly by endothelial cells, is involved in angiogenesis and mitogenesis. Myofibroblasts (myoFb) are phenotypically transformed fibroblast-like cells found at the site of myocardial infarction. Since myoFb play a role in tissue repair/remodeling at the site of infarction, and express endothelin and angiotensin II (AngII), it was interesting to investigate whether myoFb express VEGF and its receptors *de novo*, and if the expression is influenced by vasoactive peptides. Primary cultures of myoFb were isolated from 4-week-old adult rat heart infarct were used in this study. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR), utilizing primers designed to amplify known isoforms of VEGF revealed expression of two predominant forms, VEGF120 and VEGF164 and northern blot hybridization detected VEGF mRNA of 4.5 kb. VEGF actions are mediated via two major receptors, Flt-1 and KDR, and hence the expression of these receptors was investigated. Flt-1 and KDR expression in myoFb was detected by RT-PCR, RNA transcripts were confirmed by northern blot hybridization while western blot confirmed the presence of VEGF, Flt-1 and KDR proteins in myoFb. In this study AngII upregulated VEGF and Flt-1 expression in myoFb, but not KDR; this was mediated predominantly by AT1-receptor. We report for the first time that cardiac myoFb, isolated from the site of infarction express VEGF, its receptors, Flt-1 and KDR, with modulation of VEGF and Flt-1 expression by AngII. Thus, VEGF may contribute to tissue remodeling and angiogenesis at the site of infarction in an autocrine manner.

Keywords: Cardiac myofibroblasts; VEGF; Angiotensin II; Myocardial infarction

1. Introduction

Myofibroblasts (myoFb) exhibit phenotypic heterogeneity among fibroblasts expressing features of smooth muscle cells [1–4]. They take part in the growth, development, and repair of normal tissue as well as the diseases affecting many different organs, yet there are few reports available on the role of myoFb in heart remodeling during myocardial infarction [5,6]. Previously, we have shown that myoFb isolated from the site of myocardial infarction are non-endothelial cells exhibiting morphological features of fibroblasts and smooth muscle cells by expressing α-smooth muscle actin, but not factor VIII [5]. Evidence suggests that they play a central role in wound healing and matrix remodeling during post-myocardial infarction [5,7,8]. These wound healing myoFb serve as a local source for endothelins, angiotensins and their receptors [5,9–11]. Several vascular growth factors have been implicated as playing important roles in the invasive properties, proliferation, and migration phases of angiogenesis. Accumulated evidence has demonstrated that vascular endothelial growth factor (VEGF) is a key regulator for angiogenesis under various physiological and pathological conditions, such as embryonic development, wound healing, and solid tumor growth [12–18].

VEGF, also known as vascular permeability factor (VPF), is a polypeptide secreted by a large number of cells, but predominantly by endothelial cells. VEGF is a mitogen and survival factor for endothelial cells that also induces angiogenesis and vasculogenesis *in vivo* [19–23]. VEGF also shows a mitogenic effect on a few non-endothelial cell types such as retinal pigment epithelial cells, pancreatic duct cells, and Schwann cells *in vitro* [24]. VEGF family members are differentially expressed in various organs, including the

* Corresponding author.
Tel.: +1-252-744-1906, -744-2762; fax: +1-252-744-3460.
E-mail address: katwal@mail.ecu.edu (L.C. Katwa).

DOI: 10.1016/S0022-2828(03)00006-3

heart, where co-expression of VEGF and VEGF-B is predominant [25]. Alternative exon splicing of eight cassettes within a single VEGF gene results in the generation of several VEGF isoforms, among which VEGF121 and VEGF165 are predominant [24]. VEGF mediates biological functions mainly by Flt-1 and KDR/Flk-1 receptors; studies using knockout mice lacking VEGF or its receptor revealed that VEGF plays a critical role in the development and formation of blood vessel network [26]. Previous studies have identified that vasoactive peptides induce VEGF production. Chua *et al*. [27] reported that angiotensin II (AngII) acts on rat heart endothelial cells to induce VEGF mRNA in a dose and time-dependent manner. Otani *et al*. [28] reported AngII potentiation of VEGF dependent cell growth and tube formation of retinal microvascular endothelial cells through induction of the VEGF receptor KDR/Flk-1. Based on the previous reports on endothelial cells and few non-endothelial cell types, we postulated that myoFb might serve as a local source of VEGF at the site of remodeling, with AngII modulating expression of VEGF and its receptors in myoFb. Therefore, our objectives in the current investigation were to identify whether myoFb expressed VEGF and its receptors *de novo*, which may prove to be the local source of growth factors required for angiogenesis and remodeling following myocardial infarction as well as modulation of VEGF and its receptors expression by AngII.

2. Materials and methods

2.1. Materials

Protease inhibitors (leupeptin, aprotinin, pepstatin and phenylmethyl sulfonyl fluoride), AngII and secondary antibodies were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dup-753 and PD-123319 were generous gift from Du-Pont Pharmaceuticals (Wilmington, DE, USA) and Park-Davis Pharmaceuticals (Research Division, MI, USA), respectively. VEGF, Flt-1 and KDR polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Delaware Avenue, CA, USA). Tissue culture materials (antibiotics, DMEM medium, etc.), Trizol, and thermoscript one-step quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) kit were purchased from Invitrogen (Life Technologies, Grand Island, NY, USA). Enzymes and other reagents required for RT-PCR were obtained from Promega Biotech (Madison, WI, USA). β_2-microglobulin, VEGF, Flt-1, and KDR oligonucleotide primers were obtained from Synthegen (Houston, TX, USA). All other general chemicals were of reagent grade and were obtained from Fisher scientific (Atlanta, GA, USA).

2.2. Isolation of myoFb

myoFb were isolated under aseptic conditions from the border region of grossly visible scars in adult male Sprague–Dawley rats (250–300 g) 28 d after induction of myocardial infarction by ligation of the left anterior descending coronary artery [5]. Previous *in vivo* studies from our group have confirmed the presence of myoFb in these regions at 28 d of post-infarction [8]. Tissues recovered from this region were washed in saline, minced to small pieces and placed in a six well plates each containing a few drops of 20% FCS in DMEM with amphotericin and gentamycin. After 6 h, additional medium with 10% FCS was added. Cells were washed after 24 h with DMEM containing 10% FCS and medium was subsequently replaced every 2–3 d. myoFb were grown and maintained under the conditions as described previously [5,7]. After 2 weeks of culturing, cells reached confluence and were routinely split at 1:3 ratio. Early passages of myoFb, i.e. 3–4, were frozen in 10% DMSO, stored in liquid nitrogen and used when needed.

2.2.1. Treatments

Early passages of myoFb cells were grown to 80% confluence in 100 mm culture dishes using DMEM containing 10% FCS. Prior to treatment, culture media was replaced with serum-deprived (0.4% FCS) DMEM media for 12 h in order to eliminate serum growth factors influence on experimental conditions. Our preliminary studies (data not shown) indicated myoFb required 24 h treatment with 10–100 nM AngII to significantly stimulate VEGF expression, while Dup-753, AngII type 1 (AT1), and PD-123319, type 2 (AT2), receptor blockers optimally inhibited AngII influence on VEGF expression at 10 μM. Untreated myoFb cells under similar culture conditions served as controls. Optimal VEGF mRNA levels were observed in the cells treated for 3 h. Therefore, these concentrations and time intervals were used for the current investigation.

2.3. RNA isolation and purification

Total RNA was isolated from treated and untreated myoFb cells using TRIZOL method. RNA samples were further subjected to RNase-free DNase I treatment at 37 °C for 30 min, then purified with phenol chloroform and isoamyl alcohol, re-precipitated, and dissolved in nuclease-free water. RNA was quantified with GeneQuant *pro* spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and RNA integrity was confirmed by running it on a formaldehyde gel. The isolated total RNA was used in RT-PCR, real-time quantitative RT-PCR, and northern hybridization experiments.

2.3.1. cDNA synthesis

Total RNA was extracted from myoFb according to the RNA isolation and purification method as mentioned in Section 2.3. Complementary DNA (cDNA) was synthesized using Life Technologies SuperScript™ first strand synthesis kit as follows: 1 μl (10 mM) dNTP mix, 1 μl (0.5 μg/μl) oligo ($dT_{12–18}$) primers, 5 μg total RNA, the reaction volume was brought to 10 μl with DEPC water and incubated at 65 °C for 5 min followed by at least 1 min incubation on ice. Followed by 2 μl (10×) RT buffer, 4 μl (25 mM) $MgSO_4$, 2 μl (0.1 M)

Table 1
Gene specific nucleotide sequences used for PCR and real-time PCR

Gene	Primer designation	Primer sequence 5' → 3'	Amplicon length base pairs
β_2-MG	β_2-MG-5'	5'-CTCCCCTTCAAGTTGTACTCTCG-3'	250
	β_2-MG -3'	5'-GAGTGACGTGTTTAACTCTGCAAGC-3'	
VEGF	VEGF-5'	5'-CTCTACCTCCACCATGCCAAG-3'	500, 600
	VEGF-3'	5'-GGTACTCCTGGAGGATGTCCACC-3'	
KDR	KDR-5'	5'-GTACTCCAGCGACGAGGCAGGACTTTTA-3'	445
	KDR-3'	5'-TTTTATCCAGTTTCACAGAGGGCTCCATTG-3'	
Flt-1	Flt-5'	5'-TGGAAGGAGGCGAGGATTACAGTGAGA-3'	453
	Flt-3'	5'-GGTAGATTTCAGGTGTGGCATACTCTGGTG-3'	

DTT, 1 μl (40 units) RNase out were added to above reaction tube. The reaction mixture was incubated at 42 °C for 2 min and 1 μl (50 units) of SuperScript™ reverse transcriptase was added and incubated at 42 °C for 50 min. Finally, above reaction was terminated by incubating at 70 °C for 15 min. Upon completion of the reaction, the final reaction mixture volume was brought to 100 μl with distilled water. The PCR product was purified using Life Technologies kit followed by DNase-free RNase treatment and re-extraction. β_2-microglobulin (β_2-MG) was chosen as an internal control to monitor cDNA synthesis efficiency and to optimize the amount of cDNA amplified.

2.4. Polymerase chain reaction

To investigate the expression of different VEGF mRNA splice variants and its receptors (Flt-1 and KDR), PCR was performed using specific oligonucleotide primers as shown in Table 1. Total RNA was extracted from untreated (controls) as well as treated myoFb, and cDNAs were synthesized using the RT reaction as described previously. PCR amplification of cDNA from each sample was performed utilizing PCR Sigma Redtaq SuperPak™ as follows: 10× PCR buffer, 200 μM dNTP mix, 0.5 μM specific primers for VEGF, Flt-1 or KDR and β_2-microglobulin (Table 1) and 0.05 units/μl of polymerase in a final volume of 25 μl. Following an initial denaturation at 94 °C for 1 min, VEGF, Flt-1, KDR and β_2-microglobulin were subjected to 35 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 1 min, primer extension at 72 °C for 2 min and, a final extension at 72 °C for 5 min to ensure all reactions were completed. PCR products were electrophoretically size-fractionated on 1.5% agarose gels containing 0.05 μg/ml of ethidium bromide and visualized with UV-light.

2.5. Real-time quantitative RT-PCR

Thermoscript one-step quantitative RT-PCR with platinum Taq kit was used in order to synthesize cDNA and subsequent real-time quantitative RT-PCR (Cepheid Real Time PCR thermocycler, from Sunnyvale, CA, USA) reactions. The gene specific sequences of oligonucleotide primers (Table 1) were used to check the expression of respective genes with a 0.2× final concentration of SYBR Green I nucleic acid stain (Molecular Probes, Eugene, OR, USA) as the fluorophore. The final concentration of $MgSO_4$ was adjusted to 6 mM with 50 mM $MgSO_4$ in 25 μl PCR mixture according to the manufacturer's protocol. The temperature profile included an initial 30 min, at 50 °C (for cDNA synthesis), then denaturation at 95 °C for 5 min, in order to deactivate the RT and activate the thermoscript taq polymerase. This was immediately followed by 45 cycles of denaturation at 95 °C for 15 s, 60 s at 60 °C annealing and elongation with optics on for fluorescence monitoring. The specificity and purity of the amplification reaction was determined by performing a melting curve analysis. The quantification of gene expression by real-time RT-PCR was determined by comparing amplified products to a previously generated standard curve for β-actin. Briefly, this was generated by serial dilution of known quantities of total β-actin DNA using gene specific primers (Table 1) followed by quantitative real-time PCR.

2.6. Northern blot analysis

The 15 μg of isolated total RNA was denatured in a formamide/formaldehyde solution at 68 °C for 10 min and samples were resolved on 1% agarose gel electrophoresis. The gel was stained with ethidium bromide solution, photographed, washed and transferred by vacuum to a nylon membrane (MSI, Westboro, MA, USA) for 5 h. After UV crosslinking, blots were prehybridized in a buffer containing 50 mM PIPES, 100 mM NaCl, 50 mM sodium phosphate (pH 7.0), 1 mM EDTA and 5% SDS at 55 °C for 30 min. Radioactive [α-^{32}P] dCTP (3000 Ci/mmol, Amersham International, Buckinghamshire, UK) cDNA probes were generated by RT-PCR for rat VEGF, Flt-1 and KDR. The RNA blotted membranes were subsequently hybridized for 16 h at 65 °C. The membrane was washed in 1× standard saline citrate plus 5% SDS at 55 °C for 30 min, and exposed to X-ray film at –70 °C for appropriate time, then developed.

2.7. Western blotting

Treated/untreated sub-confluent myoFb were lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 0.25% NP-40, 1 mM phenylmethylsulfonylfluoride and 1× protease inhibitors). Fifty micrograms of protein was analyzed on (4–16%) gradient SDS-PAGE under denaturing conditions and electrotransferred to nitrocellulose membranes (Sigma). Non-

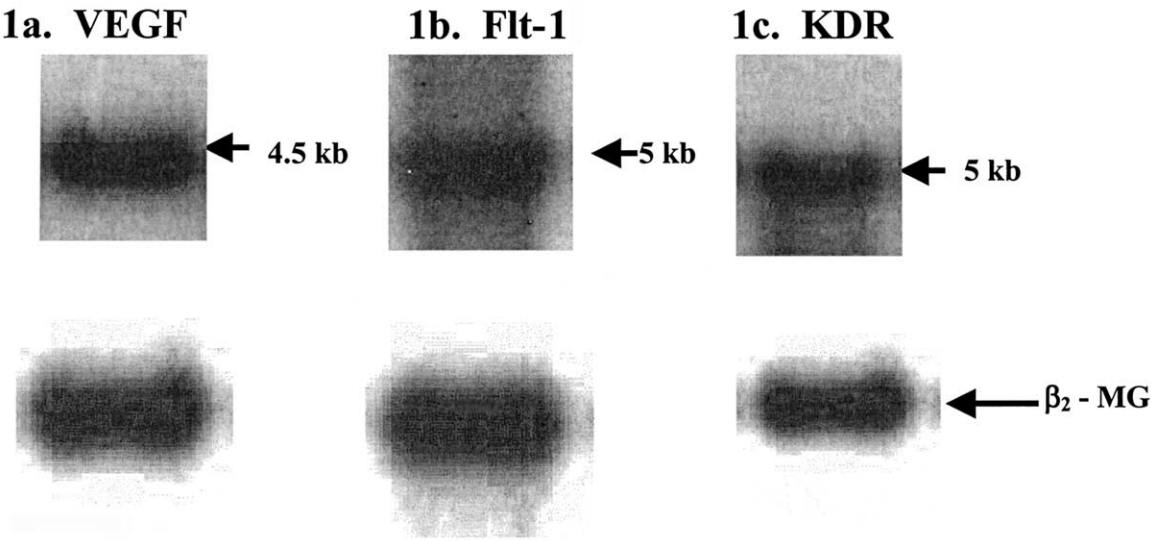


Fig. 1. Northern blot analysis demonstrating the expression of VEGF (a), Flt-1 (b) and KDR (c) mRNA in cultured myoFb; (a) shows 4.5 kb transcript of VEGF, (b,c) shows 5 kb Flt-1 and KDR transcripts respectively, and β2 Microglobulin was used as an internal control

specific protein binding was blocked by incubating the membranes with blocking solution (PBS and 5% non-fat dried milk) for 60 min, at room temperature. Polyclonal antibodies specific for VEGF/Flt-1/KDR (1:250 in PBS containing 5% non-fat dried milk) were applied to the membrane and incubated overnight at 4 °C. After rinsing with wash buffer (PBS, 0.15% Tween 20), membrane was incubated in 1:5000 diluted peroxidase-conjugated anti-mouse IgG antibody (Sigma, St. Louis, USA) for 60 min at room temperature. The detection of specific signals was performed using the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Protein concentrations were measured using the method of Bradford (BIO-RAD, Hercules, CA, USA).

2.8. Statistical analysis

Results are reported as mean ± S.E.M. for a minimum of five or six determinations each of which was performed in duplicate or triplicate. Statistical analysis was performed using one-factor ANOVA, with $P < 0.05$ considered significant.

3. Results

3.1. Expression of VEGF, Flt-1 and KDR mRNA in myoFbs

VEGF and its receptors Flt-1 and KDR gene expression was demonstrated in myoFb by northern blot (Fig. 1). The mRNA transcript of 4.5 kb was detected for VEGF (Fig. 1a) and a 5.0 kb mRNA transcript was detected for both Flt-1 and KDR, as shown in Fig. 1b,c.

3.2. Expression of VEGF, Flt-1, and KDR protein in myoFb

Protein levels were determined using western immunoblot analysis of myoFb lysate with specific anti-VEGF, anti-Flt-1 and, anti-KDR antibodies corresponding to respective protein and protein bands for VEGF (at 23 kDa), Flt-1 (at 120 and 220 kDa), and KDR (at 220 kDa), are shown in Fig. 2.

3.3. VEGF and its receptor expression in myoFb by RT-PCR

VEGF, Flt-1, and KDR mRNA expression was examined in myoFb using RT-PCR technique. PCR amplification of myoFb cDNA with VEGF, Flt-1 and KDR specific primers resulted in PCR products of the expected size for VEGF (500, 600, 1000, and 1200 bp), Flt-1 (453 bp) and KDR (445 bp), respectively (Fig. 3). Results indicated that VEGF PCR products 500, 600, 1000, and 1200 bp corresponded to VEGF 120, 164, 188 and 205, isoforms, respectively, with VEGF 120 and 164 as predominant isoforms (Fig. 3). These results clearly demonstrate the expression of four different VEGF isoforms and its receptors (Flt-1 and KDR) in myoFb.

3.4. Effect of AngII and its receptor antagonists on VEGF expression in myoFb by western blot

The objective of this study was to investigate the modulation of VEGF protein expression in myoFb treated with and without AngII and its receptor antagonists (Fig. 4). Our results revealed that AngII upregulated the VEGF expression in myoFb and the influence of AngII was completely abrogated when treated along with Dup-753 whereas only incomplete inhibition was observed when treated simultaneously with PD-123319. The decline of VEGF expression by Dup-753 was statistically significant ($P < 0.05$) but not by PD-

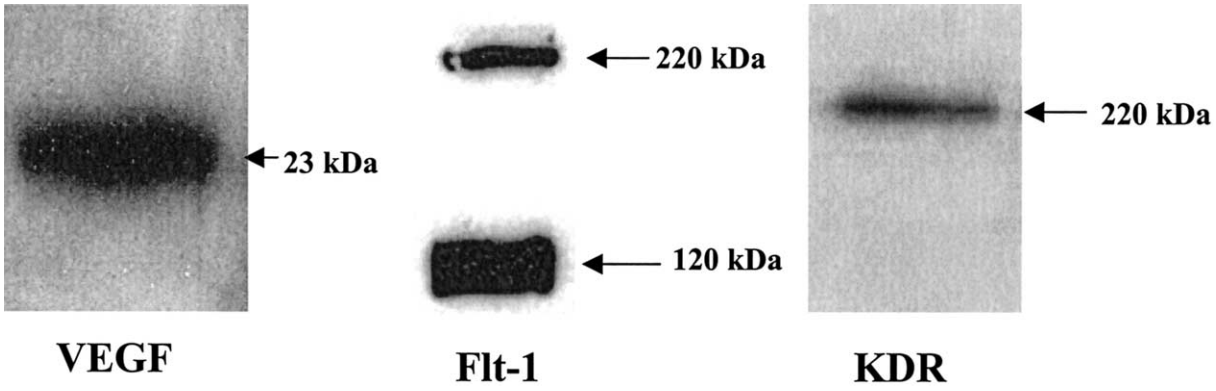


Fig. 2. Western blot analysis of VEGF, Flt-1, and KDR expression using 50 μg of total protein from cultured myoFb. Electrophoreses was performed under denaturing conditions on a 4–16% gradient sodium dodecyl sulfate-polyacrylamide gel. After transblotting to nitro-cellulose membrane, proteins were revealed by a specific polyclonal antibody (1:250) and ECL detection. VEGF migrated to 23 kDa, KDR to 220 kDa, and Flt-1 to 220 and 120 kDa

123319 ($P < 0.8$), demonstrating that the AngII influence on VEGF expression mediated mainly by AT1 receptor.

3.5. Effect of AngII and its receptor antagonists on Flt-1 and KDR expression in myoFb by western blot

Experiments were conducted to study AngII effect on Flt-1 and KDR protein expression. In these experiments, myoFb were treated with and without AngII and its receptor antagonists and results are shown in Fig. 5. Our results demonstrated that AngII upregulates the Flt-1 protein expression in myoFb which was significantly inhibited by Dup-753 (Fig. 5, $*P < 0.05$) but not by PD-123319 (Fig. 4, lane 2, $P < 0.8$). This study clearly demonstrates AngII upregulation of Flt-1 expression is mediated predominantly by AT1 receptor, while AngII alone or in combination with antagonists did not show any significant effect on KDR protein expression (Fig. 6).

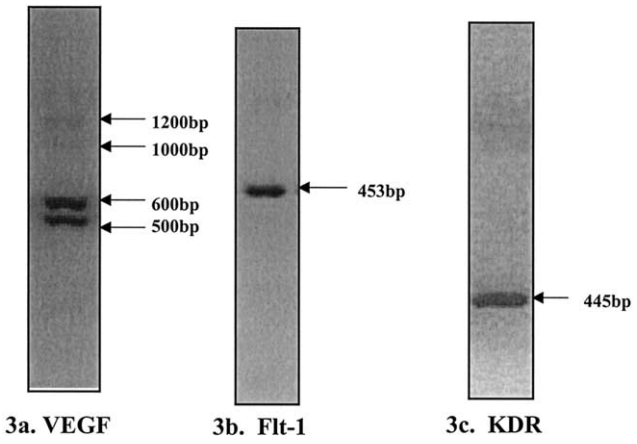
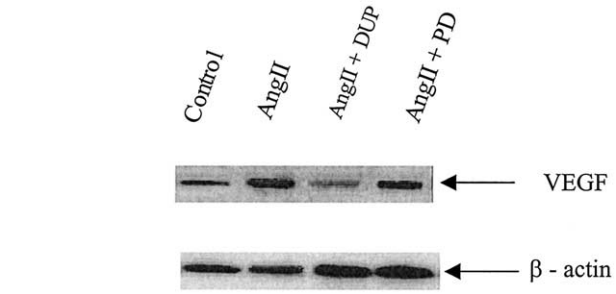


Fig. 3. The expression of VEGF and its receptors (Flt-1 and KDR) by RT-PCR in myoFb. Total RNA from myoFb was used for cDNA synthesis by RT reaction and amplified by PCR using specific primers. (a) reveals the expression of different splice variants of VEGF (1200, 1000, 600, and 500 bp). (b, c) both show 453 and 445 bp PCR products corresponding to Flt-1 and KDR, respectively



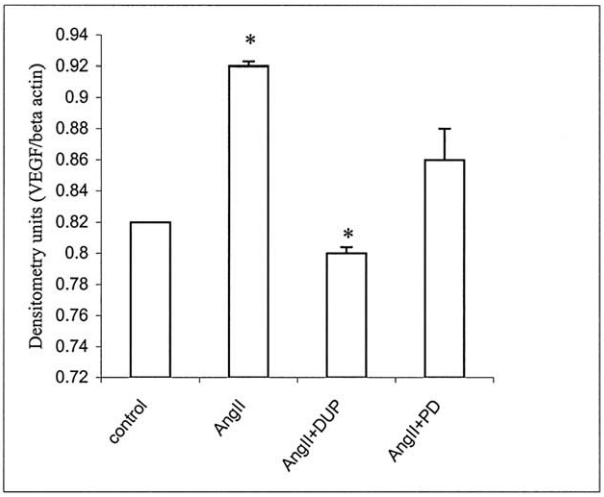


Fig. 4. Western blot analysis showing the effects of AngII in the absence or presence of its receptor antagonists (Dup-753 and PD-123319) on VEGF protein in cultured cardiac myoFb. Untreated cells (lane 1); cells treated with 10^{-7} M AngII (lane 2); 10^{-5} M Dup-753 and 10^{-7} M AngII (lane 3); 10^{-5} M PD-123319 and 10^{-7} M AngII (lane 4) are shown. Lower panel (histogram) represents the averages of densitometric scan of six ($n = 6$) experiments and above panel shows one of the representative figures. VEGF protein was normalized to internal control (β-actin). Results show the mean ± S.E.M. of VEGF to internal control with $*P < 0.05$ indicating that data is statistically significant

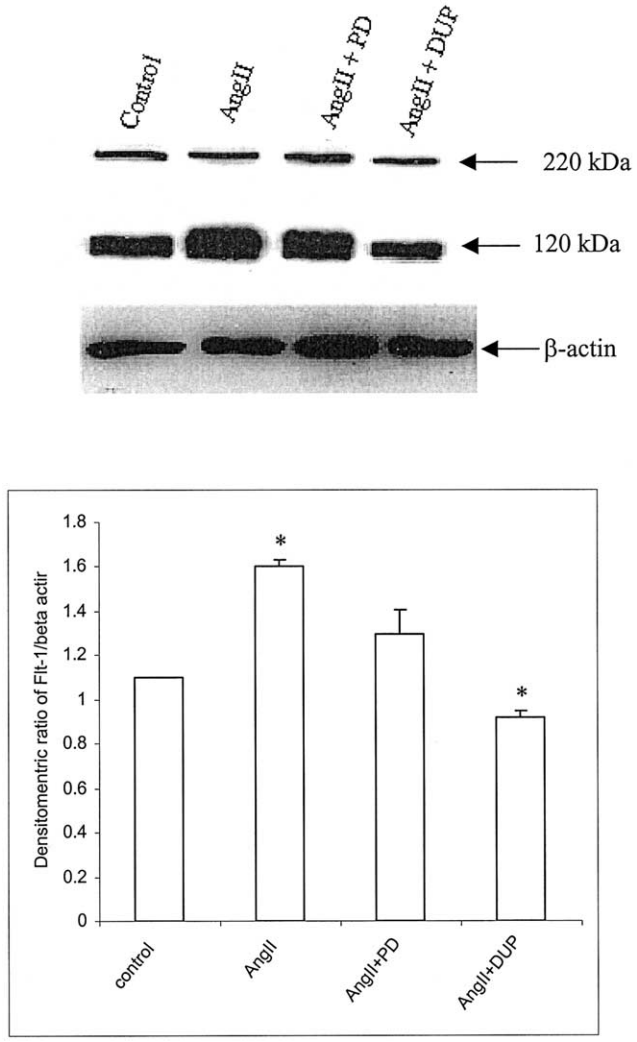


Fig. 5. Western blot analysis of AngII and its receptor antagonists (Dup-753 and PD-123319) on Flt-1 in cultured cardiac myoFb. Cells were treated with or without AngII (10^{-7} M) and its receptor antagonists (10^{-5} M) for 24 h in serum-free medium. Samples were loaded in the following order; lane 1 control, 2 AngII, 3 AngII and PD-123319, and 4 AngII and Dup-743. Lower panel shows a histogram results from six blots corresponding to different treatments ($n = 6$, top panel shows one of the representative figure). Results show the ratios of mean ± S.E.M. of Flt-1 to internal control ($^*P < 0.05$)

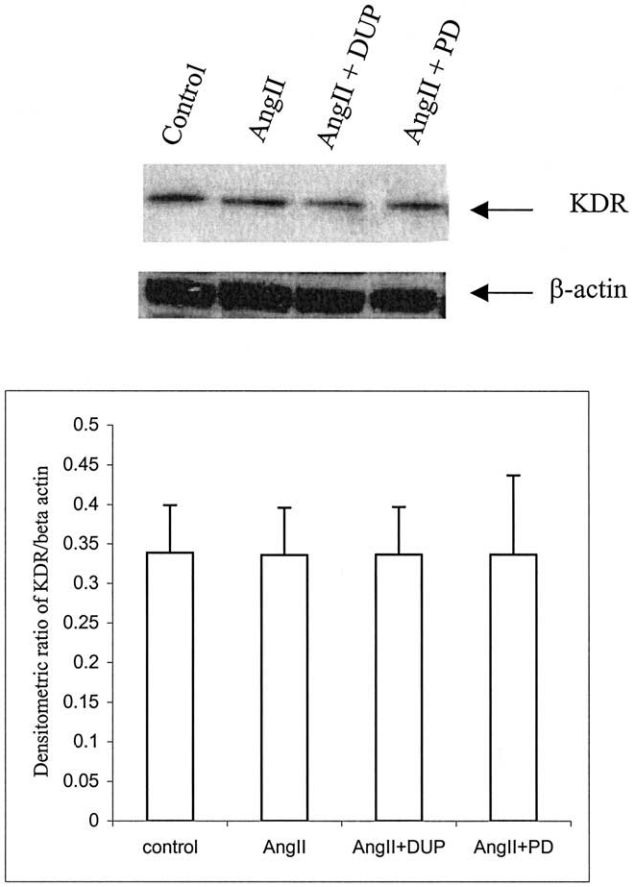


Fig. 6. Western Blot analysis shows the influence of AngII and its receptor antagonists (Dup-753 and PD-123319) on KDR expression in cultured cardiac myoFb. Cells were treated with or without AngII (10^{-7} M) and its receptor antagonists (10^{-5} M) for 24 h in serum-free medium. Samples were loaded in the following order; lane 1 control, 2-AngII, 3-AngII and Dup-743, 4-AngII and PD-123319. Lower panel is a histogram of western blots showing the ratio of KDR to the β-actin at different treatments, results are mean ± S.E.M. ($n = 4$, top panel indicates one of the representative figure)

3.6. Modulation of VEGF gene expression by AngII in myoFb

3.6.1. Semiquantitative RT-PCR

Effect of AngII on VEGF expression was also studied using semiquantitative RT-PCR technique. myoFb treated with AngII showed increased VEGF gene expression compared to control (untreated). AngII influence was blocked when treated along with Dup-753, but not with PD-123319. These results emphasize that modulation of VEGF by AngII is mainly through AT1 receptor (Fig. 7a).

3.6.2. Real-time quantitative RT-PCR

The results on VEGF gene expression in myoFb treated with AngII showed significant ($^*P < 0.05$) increase in VEGF (144%) compared to untreated controls (baseline; control as 100%). As shown in Fig. 7b, Dup-753 (AT1 receptor blocker) treatment along with AngII (AngII + Dup-753) significantly ($^*P < 0.05$) inhibited AngII effect on VEGF gene expression (13% with reference to baseline 144–87%). Whereas, PD-123319 and AngII treated myoFb showed partial abrogation of AngII influence on VEGF expression when compared to AngII alone treated cells. However, AT2 receptor mediated VEGF expression by AngII was marginal ($P < 0.8$) compared to AT1. These results suggest AngII upregulation of VEGF expression mostly via AT1.

Results from real-time RT-PCR and semiquantitative RT-PCR confirm the upregulation of VEGF by AngII and also a role for AT1 receptor in this process.

3.7. Influence of AngII on Flt-1 & KDR gene expression in myoFb

3.7.1. Semiquantitative RT-PCR

Fig. 8a shows that AngII and its receptor antagonist's influence on Flt-1 gene expression in myoFb by semiquanti-

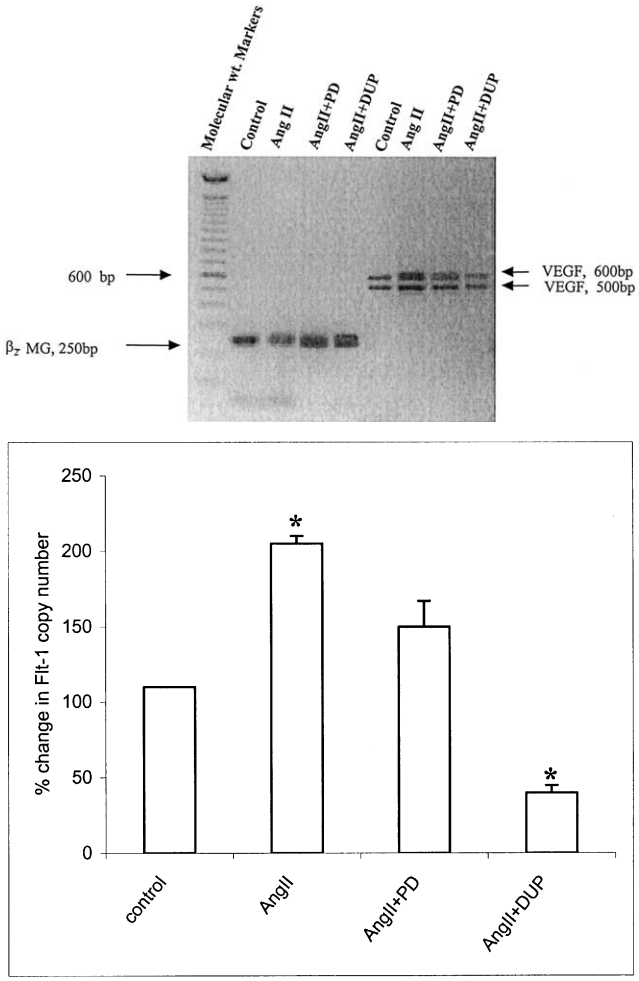


Fig. 7. (a) RT-PCR analysis of VEGF mRNA isolated from AngII and its receptor antagonists treated cultured myoFb. Total cellular mRNA was reverse-transcribed into cDNA and amplified with VEGF and β2-MG-specific primers. β2-MG was used as an internal control and PCR products were of the predicted size: VEGF, 500 and 600 bp; β2-MG, 250 bp. (b) Histogram showing the effect of AngII receptor antagonists' Dup-753 and PD-123319 on the induction of VEGF mRNA in rat cardiac myoFb. Cells were treated with or without AngII (10^{-7} M) and its receptor antagonists (10^{-5} M) for 3 h in serum-free medium. Total 200 ng of total RNA was used in each case for real-time quantitative RT-PCR with specific primers for VEGF as shown Table 1. Gene expression was quantified as percent change in copy number (*$P < 0.05$) compared to β-actin standard curve. Values are the mean ± S.D. from six independent experiments

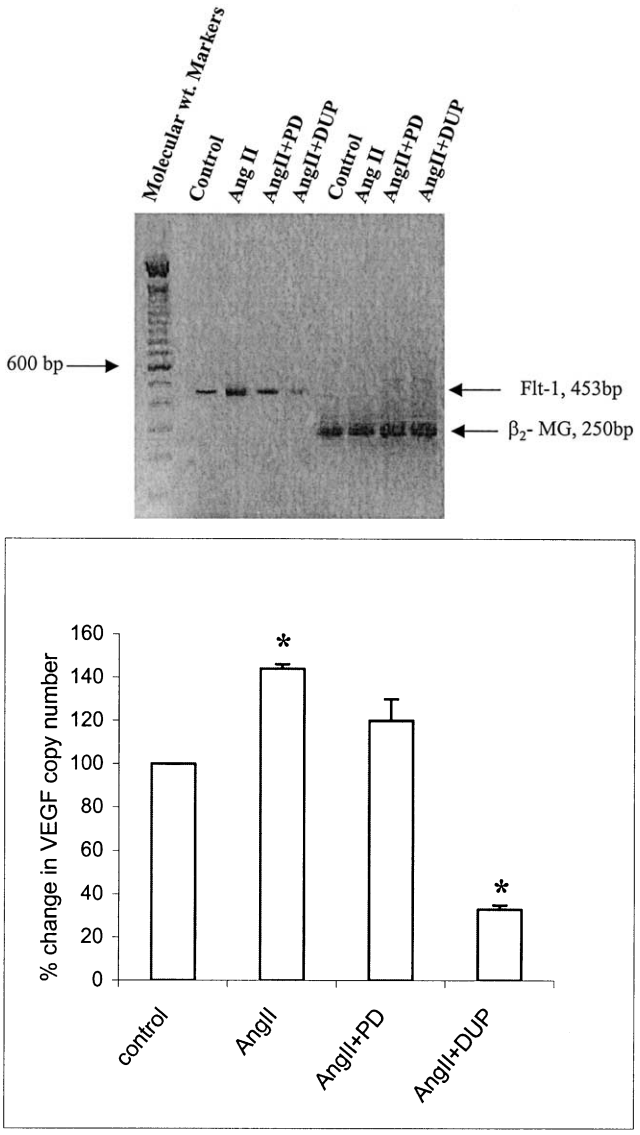


Fig. 8. (a) RT-PCR analysis of Flt-1 mRNA isolated from AngII and its receptor antagonists treated cultured myoFb. Total cellular RNA was reverse-transcribed into cDNA and amplified with VEGF and β_2-MG-specific primers. β_2-MG was used as an internal control and PCR amplified products were of the predicted size: Flt-1, 453 bp; β_2-MG, 250 bp.(b) A histogram showing the effects of AngII receptor antagonists Dup-753 and PD-123319 on the induction of Flt-1 mRNA in rat cardiac myoFb. Cells were treated with or without AngII (10^{-7} M) and its receptor antagonists (10^{-5} M) for 3 h in serum-free medium. After RNA extraction, 200 ng of total RNA was used for real-time quantitative RT-PCR, amplified with specific primers for Flt-1 (Table 1), and quantified by comparing percent change in copy number from six mean ± S.D. (*$P < 0.05$) independent experiments to β-actin standard curve.

tative RT-PCR. myoFb treated with AngII alone, showed induction of Flt-1 expression, while Dup-753 + AngII treatment blocked AngII influence on Flt-1 gene expression, but not with PD-123319. These results clearly demonstrate that AngII upregulates Flt-1 expression through AT1 receptor.

3.7.2. Real-time quantitative RT-PCR

In these experiments, real-time PCR results demonstrated over expression of the Flt-1 receptor gene in myoFb samples treated with AngII (increased to 220%) when compared to untreated controls (100%) (Fig. 8b). When myoFb were treated with Dup-753 (AT1 receptor antagonist) and AngII (AngII + Dup-753), a significant (*$P < 0.05$) decrease in AngII effect on Flt-1 gene expression was observed when compared to control (100–35%) and AngII treated alone (220–35%). When myoFb treated with PD-123319 (AT2 receptor antagonist) and AngII, partial abrogation of AngII effect on Flt-1 expression was observed when compared to AngII treated myoFb (220–150%, control baseline = 100%). These results suggest that AngII influence on Flt-1 expression was predominantly mediated through AT1 receptor. In these groups, KDR gene expression was not altered in any

treatments and no significant change in KDR gene expression was noticed in myoFb indicating that AngII predominantly influences VEGF and the Flt-1 receptor, but not the KDR receptor.

Results from real-time RT-PCR and semiquantitative RT-PCR experiments were similar and confirmed that upregulation of Flt-1 by AngII and a role for AT1 receptor.

4. Discussion

myoFb are ubiquitous cells with similar properties and functions playing important roles in growth and development, wound repair, and disease. These cells exhibit characteristic features of both fibroblasts and smooth muscle cells and are hence termed as myoFb [5–7,29]. Angiogenesis is implicated in the pathogenesis of a variety of disorders. The role of VEGF in developmental and pathological angiogenesis is well documented [24]. VEGF (also known as VEGF-A or VPF) is a secreted, heparin binding 45 kDa glycoprotein that stimulates angiogenesis in numerous physiological and pathological settings [21]. VEGF is a potent mitogen for endothelial cells and is chemotactic for vascular smooth muscle cells [30]. Expression of vasoactive peptides such as AngII and TGF-β_1 by myoFb and their role in cardiac remodeling has been demonstrated [5,7]. Association of angiogenic factors like VEGF to wound healing myoFb may have implications in regulation of angiogenesis during repair/remodeling process. VEGF production in myoFb raises intriguing questions regarding its role in angiogenesis following myocardial infarction. The present study demonstrates for the first time that cardiac myoFb express VEGF and its receptors, and their expression was markedly upregulated by AngII.

The first objective of this study was to determine if myoFb express mRNAs for VEGF/VEGF isoforms, their receptors Flt-1 and KDR, and to elaborate their corresponding proteins; this was demonstrated by a variety of techniques. Northern hybridization results show the expression of 4.5 kb VEGF transcript in cardiac myoFb. This is in agreement with the earlier studies of Olofsson *et al*. [25], who reported major transcripts of 3.7, 4.5 kb VEGF along with a 1.4 kb VEGF-B, the most abundantly expressed transcripts in heart, skeletal muscle, pancreas and prostrate endothelial cells. In our SDS-PAGE experiments, we detected VEGF and VEGF-B expression (data not shown). DiSalvo *et al*. [31] reported that pure VEGF homodimer migrates at its sub-unit mass of 27 kDa on reducing SDS-PAGE.

myoFb expressed mRNA for the four VEGF isoforms: VEGF120, VEGF164, VEGF188, and VEGF205. Rat VEGF gene contains eight *exons*, VEGF120 lacks *exons 6* and *7*, VEGF164 lacks *exon 6*, and VEGF188 incorporates all eight *exons*. The major known functional difference among the various VEGF isoforms is their ability to bind to heparin and heparin sulfate proteoglycans distributed on cellular surfaces, within extracellular matrixes, and basement membranes, this ability of VEGF is believed to be imparted mainly by *exon 6*. Recent RT-PCR findings by Chen *et al*. [32] revealed a similar profile of VEGF splice variants in a co-culture model developed to study the effect of VEGF produced by rat glomerular epithelial cells on the permeability of endothelial cells *in vitro*.

Two receptor tyrosine kinases, Flt-1, and KDR mRNA transcripts of size 5.0 kb were expressed in cardiac myoFb cells. Yamane *et al*. [33] reported a 4.7 kb Flt-1 transcript in rat liver endothelial cells and Wen *et al*. [34] reported a 6.0 kb of KDR transcript in rat retinal endothelial cells. The Flt-1 protein under reducing conditions migrated at 120 and 220 kDa in SDS-PAGE analysis and KDR migrated at 220 kDa in cardiac myoFb. In mouse vascular endothelial cells, Zachary *et al*. [35] reported a 180 kDa size of Flt-1 protein and 230 kDa of KDR protein.

Angiogenesis is the growth of new blood vessels from preexisting ones and is a complex process regulated by numerous factors. Among these factors, AngII, the main effector peptide of the renin-angiotensin system, may contribute to vessel growth regulation. AngII acts by binding to both its isoform receptors, angiotensin type 1 (AT1) and type 2 (AT2), yet induces activation of endothelial and smooth muscle cell growth mainly by the AT1 receptor [36]. AngII, a vasoactive peptide, also increases the expression and synthesis of extracellular matrix proteins. In infracted rat heart, quantitative *in vitro* autoradiography localized high density angiotensin converting enzyme (ACE) and AngII receptor binding to the site of tissue repair [8,37,38]. Previously, we have identified that cultured myoFb express various components involved in AngII generation [5] and elucidation of these effector mechanisms regulated by this vasoactive peptide may contribute to understanding of VEGF interaction with its receptors.

The second objective of the present study was determination of the effect of AngII on VEGF and its receptors expression in myoFb. Our data demonstrates that AngII induced cardiac myoFb to increase production of VEGF mRNA and protein. AngII influence on VEGF expression was completely inhibited by Dup-753, and partially inhibited by PD-123319. These studies suggest that induction of VEGF expression by AngII was mediated mainly by AT1. Gruden *et al*. [39] reported AngII increased VEGF protein production in human mesangial cells, which was completely abolished by Losartan (Dup-753), a specific AT1 receptor antagonist. Tamarat *et al*. [36] also reported in ischemic hind limb that AngII enhanced VEGF protein content and this effect was hampered by AT1 receptor blockade. Chua *et al*. [27] reported that AngII acts on rat heart endothelial cells to induce VEGF mRNA in a dose and time dependant manner in rat endothelial cells. However, the induction is mainly mediated by AT1 receptors alone with no involvement of AT2 receptors.

AngII increased the mRNA and protein expression of VEGF receptor Flt-1, but not KDR. Similar to VEGF gene expression, Dup-753 significantly decreased the Flt-1 gene expression whereas PD-123319 was less efficient in blocking

AngII effect. AngII and its receptor antagonists did not have any significant effect on KDR gene expression. These findings suggest that AngII might potentiate Flt-1 gene expression mediated mainly by AT1 in cardiac myoFb cells. Otani *et al*. [28] reported similar results in retinal microcapillary endothelial cells. Similarly, their findings indicated an increase in KDR gene expression and protein synthesis after AngII treatment, which was inhibited by AT1 receptor antagonist, but not by AT2 receptor antagonist, suggesting that AngII influence on KDR expression was predominantly mediated by AT1 receptor signaling pathways.

In our previous studies [5] we have reported endogenous expression of AngII by myoFb. This endogenously expressed AngII may have influence on the basal level expression of VEGF and Flt-1 in normal myoFb. In the present work, when myoFb were treated with Dup-753 along with AngII, the expression of VEGF and Flt-1 was lower than control indicating that the effect of both exogenous AngII and endogenously expressed AngII, was completely blocked but not with PD-123319 treated samples. These studies suggest that induction of VEGF and Flt-1 expression by AngII was mediated predominantly by AT1 receptor.

The most important findings of the present study is the *de novo* expression of VEGF and its receptors (Flt-1 and KDR) and its actions are mediated through Flt-1 receptor in cardiac myoFb and local production of VEGF which may also participate in remodeling at the myocardial infarction site in an autocrine manner. The findings that AngII stimulates the gene expression and protein secretion of VEGF in cultured myoFb, suggest this peptide might be important in inducing the synthesis and secretion of VEGF in myoFb during myocardial infarction.

In conclusion, our studies demonstrate the *de novo* expression of VEGF and its receptors (Flt-1 and KDR) in cultured cardiac myoFb suggesting a possible role(s) of VEGF in angiogenesis and remodeling following myocardial infarction. AngII upregulates VEGF and its receptor Flt-1 expression. However, no significant changes were observed in KDR expression levels treated with AngII. Our findings also suggest that the AT1 receptor pathway might play a major role in regulation of VEGF in cardiac myoFb. Locally produced VEGF and its receptors by myoFb may have novel physiologic or therapeutic functions in myocardial remodeling and angiogenesis that underlie disease or wound healing processes. In depth studies are required to delineate the relative importance of VEGF and VEGF-B and their isoforms in angiogenesis/remodeling associated with myocardial infarction in cultured myoFb. Further studies are required to understand the role of AT1 and AT2 receptor mediated signaling pathways that influence the expression of VEGF and Flt-1 by AngII in myoFb.

Acknowledgements

The authors wish to thank Eric Moore and Sashidhar S. Nakerakanti for their technical assistance. This work was supported in part by National Institute of Health grant, NHLBI, R01 HL- 60047 (LCK), American Heart Association, National Grant-in-aid # 96012350 (LCK), and American Heart Association, postdoctoral grant award # 0225555U (VC).

References

[1] Komuro T. Re-evaluation of fibroblasts and fibroblast-like cells. Anat Embryol 1990;182:103–12.
[2] Sappino AP, Schurch W, Gabbiani G. Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. Lab Invest 1990;63:144–61.
[3] Fries KM, Blieden T, Looney RJ, Sempowski GD, Silvera MR, Willis RA, et al. Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis. Clin Immunol Immunopathol 1994;72:283–92.
[4] Schmitt-Graff A, Desmouliere A, Gabbiani G. Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity. Virchows Arch 1994;425:3–24.
[5] Katwa LC, Campbell SE, Tyagi SC, Lee SJ, Cicila GT, Weber KT. Cultured myofibroblasts generate angiotensin peptides *de novo*. J Mol Cell Cardiol 1997;29:1375–86.
[6] Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. Am J Physiol 1999;277:C1–9.
[7] Campbell SE, Katwa LC. Angiotensin II stimulated expression of transforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts. J Mol Cell Cardiol 1997;29:1947–58.
[8] Weber KT, Sun Y, Katwa LC. Myofibroblasts and local angiotensin II in rat cardiac tissue repair. Int J Biochem Cell Biol 1997;29:31–42.
[9] Katwa LC. Role of endothelins in cardiovascular disease. In: Gupta SK, editor. Pharmacology and therapeutics in the new millennium. New Delhi, India: Narosa Publishing House; 2001. p. 25–36.
[10] Tzanidis A, Lim S, Hannan RD, See F, Ugoni AM, Krum H. Combined angiotensin and endothelin receptor blockade attenuates adverse cardiac remodeling post-myocardial infarction in the rat: possible role of transforming growth factor beta. J Mol Cell Cardiol 2001;1(;33):969–81.
[11] Staufenberger S, Jacobs M, Brandstatter K, Hafner M, Regitz-Zagrosek V, Ertl G, et al. Angiotensin II type 1-receptor regulation and differential trophic effects on rat cardiac myofibroblasts after acute myocardial infarction. J Cell Physiol 2001;187:326–35.
[12] Breier G, Albrecht U, Sterrer S, Risau W. Expression of vascularendothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development 1992;114:521–32.
[13] Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, et al. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 1993;72:835–46.
[14] Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996;380:435–9.
[15] Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996;380:439–42.
[16] Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem 1995;270:12607–13.
[17] Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. Nature 1992;359:845–8.
[18] Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A. Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. Nature 1994;367:576–9.

[19] Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1995;1: 1024–8.

[20] Yuan F, Chen Y, Dellian M, Safabakhsh N, Ferrara N, Jain RK. Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. Proc Natl Acad Sci USA 1996;93:14765–70.

[21] Ferrara N, Davis-Smith T. The biology of vascular endothelial growth factor. Endocr Rev 1997;18:4–25.

[22] Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, et al. VEGF regulates endothelial cell survival by the PI3-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 1998;273:30336–43.

[23] Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 1999;103:159–65.

[24] Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am J Physiol Cell Physiol 2001;280: C1358–1366.

[25] Olofsson B, Pajusola K, Kaipainen A, von Euler G, Joukov V, Saksela O, et al. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci USA 1996;93: 2576–81.

[26] Shalaby F, Ho J, Stanford WL, Fisher KD, Schuh AC, Schartz L, et al. A requirement for Flk-1 in primitive and definitive hematopoiesis and vasculogenesis. Cell 1997;89:981–90.

[27] Chua CC, Hamdy RC, Chua BH. Upregulation of vascular endothelial growth factor by angiotensin II in rat heart endothelial cells. Biochim Biophys Acta 1998;1401:187–94.

[28] Otani A, Takagi H, Suzuma K, Honda Y. Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. Circ Res 1998;82:619–28.

[29] Roy SG, Nozaki Y, Phan SH. Regulation of alpha-smooth muscle actin gene expression in myofibroblast differentiation from rat lung fibroblasts. Int J Biochem Cell Biol 2001;33:723–34.

[30] Bausero P, Ben-Mahdi M, Mazucatelli J, Bloy C, Perrot-Applanat M. Vascular endothelial growth factor is modulated in vascular muscle cells by estradiol, tamoxifen, and hypoxia. Am J Physiol Heart Circ Physiol 2000;279:H2033–2042.

[31] DiSalvo J, Bayne ML, Conn G, Kwok PW, Trivedi PG, Soderman DD, et al. Purification and characterization of a naturally occurring vascular endothelial growth factor-placenta growth factor heterodimer. J Biol Chem 1995;270:7717–23.

[32] Chen H, Treweeke AT, West DC, Till KJ, Cawley JC, Zuzel M, et al. *In vitro* and *in vivo* production of vascular endothelial growth factor by chronic lymphocytic leukemia cells. Blood 2000;96:3181–7.

[33] Yamane A, Seetharam L, Yamaguchi S, Gotoh N, Takahashi T, Neufeld G, et al. A new communication system between hepatocytes and sinusoidal endothelial cells in liver through vascular endothelial growth factor and Flt tyrosine kinase receptor family (Flt-1 and KDR/Flk-1). Oncogene 1994;9:2683–90.

[34] Wen Y, Edelman JL, Kang T, Zeng N, Sachs G. Two functional forms of vascular endothelial growth factor receptor-2/Flk-1 mRNA are expressed in normal rat retina. J Biol Chem 1998;273:2090–7.

[35] Zachary I, Gliki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc Res 2001;49:568–81.

[36] Tamarat R, Silvestre JS, Kubis N, Benessiano J, Duriez M, deGasparo M, et al. Endothelial nitric oxide synthase lies downstream from angiotensin II-induced angiogenesis in ischemic hind limb. Hypertension 2002;39:830–5.

[37] Weber KT, Sun Y, Katwa LC, Cluetjens JP. Tissue repair and angiotensin II generated at sites of healing. Basic Res Cardiol 1997;92: 75–8.

[38] Sun Y, Weber KT. Fibrosis and myocardial ACE: possible substrate and independence from circulating angiotensin II. J Card Fail 1994;1: 81–9.

[39] Gruden G, Thomas S, Burt D, Zhou W, Chusney G, Gnudi L, et al. Interaction of angiotensin II and mechanical stretch on vascular endothelial growth factor production by human mesangial cells. J Am Soc Nephrol 1999;10:730–7.