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Doxazosin Inhibits Human Vascular Endothelial Cell Adhesion, Migration, and Invasion

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Abstract The quinazoline-derived α1-adrenoceptor antagonists, doxazosin and terazosin have been recently shown to induce an anoikis effect in human prostate cancer cells and to suppress prostate tumor vascularity in clinical specimens [Keledjian and Kyprianou, 2003]. This study sought to examine the ability of doxazosin to affect the growth of human vascular endothelial cells and to modulate vascular endothelial growth factor (VEGF)-mediated angiogenesis. Human umbilical vein endothelial cells (HUVECs) were used as an in vitro model to determine the effect of doxazosin on cell growth, apoptosis, adhesion, migration, and angiogenic response of endothelial cells. The effect of doxazosin on cell viability and apoptosis induction of human endothelial cells, was evaluated on the basis of trypan blue and Hoechst 33342 staining, respectively. Doxazosin antagonized the VEGF-mediated angiogenic response of HUVEC cells, by abrogating cell adhesion to fibronectin and collagen-coated surfaces and inhibiting cell migration, via a potential downregulation of VEGF expression. Furthermore there was a significant suppression of in vitro angiogenesis by doxazosin on the basis of VEGF-mediated endothelial tube formation ($P < 0.01$). Fibroblast growth factor-2 (FGF-2) significantly enhanced HUVEC cell tube formation ($P < 0.01$) and this effect was suppressed by doxazosin. These findings provide new insight into the ability of doxazosin to suppress the growth and angiogenic response of human endothelial cells by interfering with VEGF and FGF-2 action. This evidence may have potential therapeutic significance in using this quinazoline-based compound as an antiangiogenic agent for the treatment of advanced prostate cancer. J. Cell. Biochem. 94: 374–388, 2005.

Key words: anoikis; VEGF; endothelial cells; prostate cancer; adhesion; fibronectin; migration; angiogenesis

Prostate cancer progression to advanced disease is characterized by androgen-independence and by anchorage-independence (invasion and metastasis) [Folkman, 1995]. Skeletal tissue represents an inviting target for the metastatic spread of prostate cancer cells to the bone via a complex interplay of prostate tumor cells and endothelial cells. Thus simultaneous targeting of apoptotic and angiogenic pathways, holds promising therapeutic value for the treatment of advanced prostate cancer [Bruckheimer and Kyprianou, 2000; van Moorselaar and Voest, 2002].

Epithelial and endothelial cells require attachment to the extra-cellular matrix (ECM) for cell survival and upon loss of cell adhesion to ECM, cells undergo detachment-induced apoptotic cell death, termed anoikis [Frisch and Francis, 1994; Ruoslahti and Reed, 1994]. Reinstitution of apoptosis pathways in prostate tumors is of high significance, since resistance to apoptosis is critical in conferring therapeutic failure to treatment [Bruckheimer and Kyprianou, 2000]. Angiogenesis is a complex multistep process involving close orchestration

Abbreviations used: HUVEC, human umbilical vascular endothelial cell; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor-2; TGF-β, transforming growth factor-β; FAK, focal adhesion kinase; BPH, benign prostatic hyperplasia.

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of endothelial cells, extracellular matrix, and soluble factors. Invasion and metastasis are mechanistically closely allied processes, both involving changes in the physical coupling of cells to their microenvironment and activation/degradation of ECM [Folkman, 1995; van Moorselaar and Voest, 2002]. During angiogenesis cells are in a dynamic state and lack firm attachment to the extracellular matrix, rendering them vulnerable to anoikis [Frisch and Francis, 1994; Folkman, 1995]. Metastatic cells must detach from the primary tumor, degrade, or remodel the basement membrane and intravasate. Attachment to the metastatic site proceeds via tissue-specific microvessel cell adhesion molecules or by allowing the newly-residing tumor cells to respond to transendothelial growth factors [van Moorselaar and Voest, 2002]. A therapeutically promising approach is to target angiogenesis using pharmacological agents, that prevent neovascularization, inhibit cell proliferation and migration of endothelial cells, or by direct targeting of the tumor vasculature [Folkman, 1995; O'Reilly et al., 1997].

The α1-adrenoceptor antagonists are in clinical use as anti-hypertensive agents and for the treatment of symptomatic benign prostate hypertrophy (BPH) and long-term relief of lower urinary tract symptoms (LUTS) [Kirby and Pool, 1997]. The therapeutic benefit of these agents has been initially attributed to their ability to induce smooth muscle or vascular muscle relaxation. A growing body of evidence has documented that the piperazinyl quinazoline-based α1-adrenoceptor antagonists, doxazosin, and terazosin, have additional actions by exerting a potent apoptotic effect against prostate smooth muscle, benign and malignant epithelial cells [Kyprianou et al., 1988; Chon et al., 1999; Kyprianou and Benning, 2000] via an α1-adrenoceptor-independent mechanisms [Benning and Kyprianou, 2002]. Furthermore, terazosin has been shown to suppress prostate tissue vascularity [Keledjian et al., 2001], by inhibiting cell adhesion and migration of prostate cancer cells [Keledjian and Kyprianou, 2003; Pan et al., 2003]. Molecular characterization of the underlying mechanisms revealed that doxazosin-mediated apoptosis in human prostate cancer cells involves activation of latent apoptotic machinery via effector (Smad) activation of TGF-β1 signaling and IκB induction [Partin et al., 2003]. Additional signaling events involving disruption of cell attachment to the extracellular matrix and subsequent induction of anoikis seem to also be involved [Keledjian and Kyprianou, 2003] in a molecular cross-talk of the quinazolines' cell death actions. Serious consideration should thus be given to such FDA-approved agents for prostate cancer therapy due to their ability to inhibit angiogenesis via anoikis.

Endothelial cell activation interferes with several responses in prostate cancer cells in vitro (migration, apoptosis) and in vivo (microvascular function, angiogenesis) [van Moorselaar and Voest, 2002]. Endothelial cell adhesion (on the substratum by integrins) is one of the principal requirements for cell migration, via recruitment of the fibronectin receptor $\alpha_5\beta_1$ for angiogenesis [Meredith et al., 1993; Acollo and Pepper, 1999]. Primary endothelial cells are anchorage-dependent, and disruption of integrin-ECM interactions induces human umbilical vein endothelial cell (HUVEC) death [Frisch and Francis, 1994; Ruoslahti and Reed, 1994]. Anoikis prevents shed of epithelial cells from colonizing elsewhere, thus protecting against metastasis [Frisch and Francis, 1994], and in the case of endothelial cells prevents tumor neovascularization. [Carmeliet et al., 1999]. Vascular endothelial growth factor (VEGF) is a potent positive regulator of both physiological and pathological angiogenesis [Ferrara and Davis-Smyth, 1997], with a target cell specificity restricted to vascular endothelial cells and a direct functional targeting of cell–matrix interactions [Brooks et al., 1994; Senger et al., 1997].

Direct angiogenesis inhibitors such as angiostatin and endostatin, prevent vascular endothelial cells from proliferating, migrating or avoiding apoptosis in response to angiogenic factors such as VEGF and basic fibroblast growth factor-2 (FGF-2) [O'Reilly et al., 1994, 1997]. Indirect angiogenesis inhibitors prevent the expression/activity of a tumor protein that activates angiogenesis, or block the expression of its receptor on endothelial cells [Tarui et al., 2001].

Ample experimental and clinical evidence established the ability of doxazosin and terazosin to induce apoptosis in prostate tumor epithelial cells [Kyprianou and Benning, 2000; Benning and Kyprianou, 2002] and inhibit of prostate tumor vascularity possibly by interfering with VEGF [Keledjian et al., 2001; Keledjian and Kyprianou, 2003; Pan et al., 2003], implicating the quinazolines as inhibitors

of angiogenesis. In this study, considering that endothelial cell migration and formation of new capillary tubes are required events in the angiogenic response, we investigated the in vitro direct effect of doxazosin on apoptosis, adhesion, migration, and invasion of human vascular endothelial cells. Our findings indicate that doxazosin treatment results in a significant induction of apoptosis and inhibition of VEGF-mediated angiogenic response of endothelial cells.

MATERIALS AND METHODS

Cell Culture

Human vascular endothelial cells (HUVEC) were cultured in endothelial basal medium (EGM-2) (Clonetics, San Diego, CA) supplemented with endothelial cell growth supplements (EGM-2) (Clonetics).

Drugs

Doxazosin mesylate (Cardura) was a generous gift from Pfizer Pharmaceuticals (New York, NY). Recombinant human VEGF was purchased from R&D Systems, Inc. (Minneapolis, MN). Fibronectin was purchased from BD Biosciences (Bedford, MA).

Cell Viability Assay

Subconfluent cultures of HUVEC were exposed to increasing concentrations of doxazosin (1–50 μM) and cell viability was assessed after 24 h using the trypan blue exclusion assay [Kyprianou and Benning, 2000]. Values were expressed as the mean percent of cell viability relative to the untreated cultures. Each assay was performed (in duplicate) in three independent experiments.

Apoptosis Evaluation

Cells were plated (5×10^4 cells/well) in 6-well plates, and at subconfluency were exposed to doxazosin (5 or 15 μM). Following treatment (24 and 48 h), cells were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) and stained with 10 μg/ml Hoechst 33342 (B2261; Sigma) in the presence of 0.1% Triton X-100 (overnight at 4°C) as previously described [Bruckheimer and Kyprianou, 2001]. Cells were visualized using a Carl Zeiss Axiovert 10 fluorescent microscope (Thornwood, NY) with a UV filter (365 nm) and cells with fragmented nuclei were defined as apoptotic (×32 magnification). The apoptotic index was assessed by counting three random fields (in duplicate wells) per treatment group.

Cell Attachment Assay

HUVEC were plated on fibronectin-coated plates and after attachment doxazosin (15 μM), VEGF (15 ng/ml), FGF-2 (5 ng/ml), TGF-β (5 ng/ml) or the combination was added to the media. The attachment potential after various treatments was determined as recently described [Keledjian and Kyprianou, 2003].

Cell Migration Assay

(Wounding assay) Confluent monolayers of HUVEC cells were wounded with a toothpick as previously reported [Sato and Rifkin, 1988]. After wounding, medium was changed and VEGF (15 ng/ml), doxazosin (15 μM), or the combination of VEGF and doxazosin was added to the media. After incubation for 22 h, wounding areas were examined under light microscopy (Axiovert 10, Zeiss). Cells that had migrated to the wounded areas were counted under a microscope for quantification of cell migration. Migration was calculated as the average number of cells observed in five random high power (×400) wounded fields/per well in duplicate wells. Oncor Image Analysis system (Gaithersberg, MD) was used for capturing the images.

Tube Formation Assay

(In vitro angiogenesis assay) In vitro formation of tubular structures was studied on extracellular matrix using an angiogenesis kit as described by the manufacturer (Chemicon International, Inc., Temecula, CA). HUVEC (10×10^4 cells/well) of 96-well-plates were seeded onto ECMatrix gel (in vitro basement membrane)-coated wells in the presence or absence of doxazosin and VEGF, FGF-2, or TGF-β. Cells were treated with cytokines as single agents or each in combination (e.g. doxazosin and VEGF). After 24 h in vitro angiogenesis was assessed as formation of capillary-like structures of HUVEC, according to the manufacturer's protocol. The number of capillary-like tubes was counted in each well and the average was evaluated. Oncor Image analysis system was used under a light microscope (Axiovert 10, Zeiss).

RT-PCR Analysis

RNA was extracted from treated and untreated HUVEC cells using the TRIzol kit and

RT-PCR was performed using total RNA (1 μg) and the Ribo Clone cDNA synthesis kit (Promega Corp., Madison, WI) as previously described [Benning and Kyprianou, 2002; Keledjian and Kyprianou, 2003]. The primers for human VEGF and GAPDH were as recently described. The human KDR primers were as follows: sense, 5′-TTGAAGGTCGGAAGTCAACGGATTTGGT-3′; antisense, 5′CATGTGGGCCATGAGGTCC-ACCAC-3′. GAPDH expression was used as a loading and integrity control. The RT-PCR products were electrophoretically analyzed through 1% (w/v) agarose and visualized by ethidium bromide staining.

Western Blotting

Cell lysates were prepared from doxazosin treated (15 μM) cultures of HUVEC cells for 0, 6, 12, and 24 h. Cells were harvested with trypsin/EDTA (Clonetics) and lyzed with RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 0.5% deoxycholic acid, 1% NP-40 and Complete Mini [Roche Molecular Biochemicals, Mannheim, Germany] as a protease inhibitor). Total protein content was quantitated using BioRad protein Assay dye and protein samples (30 μg) were subjected to electrophoretic analysis through 15% sodium dodecyl sulphate (SDS)/polyacrylamide gel and transferred to Hybond P membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were incubated overnight with the caspase-3 monoclonal antibody (Pharmigen, CA), and the FAK monoclonal antibody (Upstate Biotechnology, Lake Placid, NY). Membranes were subsequently washed and incubated with species-specific horse-radish peroxidase (HRP)-labeled secondary antibodies. Signal detection was achieved using the enhanced chemilluminescence system (ECL; Amersham Pharmacia Biotech) and autoradiography with X-ray film. Densitometric analysis was performed using the Scion Image program (Scion Corp., Frederick, MD) and all bands were normalized to α-actin expression (CP01; CalBiochem, San Diego, CA).

Enzyme-Linked Immunosorbent Assays (ELISA)

For the determination of VEGF and FGF-2 levels secreted by the endothelial cells ELISA assays were applied. Subconfluent monolayers of HUVEC cells in 96-well plates (10×10^4 cells/well) were exposed to doxazosin (15 μM) for 6, 12, 24, 48, and 72 h (each time point in duplicate) and the media were collected and stored at −20°C. The concentration of the two cytokines (VEGF and FGF-2) in the culture media from untreated control and doxazosin-treated cells was evaluated using ELISA kits specific for human VEGF and FGF-2, respectively (Chemicon International, Inc.) according to the manufacturer's instructions. Two independent experiments were performed in duplicate and the mean value ± standard error of the mean (SEM) was determined.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed to determine the statistical difference between values among the different groups. Statistical analysis was performed using the Microcal Origin statistical program. All data are represented as average values +/− SEM (standard error of the mean). A P-value of less than 0.05 was considered statistically significant.

RESULTS

Doxazosin results in a dose-dependent loss of cell viability of HUVEC after 24 h of treatment (Fig. 1). At concentrations of doxazosin as low as 1 μM, 10% loss of cell viability is observed and at 15 μM there is more than 30% cell death. Morphological induction of apoptosis was detected using the Hoechst staining in doxazosin-treated HUVECs; apoptotic cells with fragmented nuclei were visualized by fluorescence microscopy. The results summarized on Table I indicate a significant increase in the number of apoptotic cells within 24 h of exposure to doxazosin ($P < 0.01$), with a further increase in apoptosis after 48 h. The apoptotic nature of the effect of doxazosin against vascular endothelial cells was further confirmed by Western blot analysis of caspase-3 expression/activation (Fig. 2). Increased protein expression of pro-caspase-3 accompanied by detection of the activated fragment (17 kDa) was observed after 6 and 12 h of doxazosin treatment (Fig. 2), preceding the massive apoptosis manifestation (Table I).

Endothelial cell adhesion (attachment and spreading) on the substratum by integrins is one of the principal requirements for cell migration [Gumbiner, 1996; Lauffenburger and Horwitz, 1996], recruiting the fibronectin receptor $\alpha_5\beta_1$ for angiogenesis [Brooks et al., 1994; Clark and

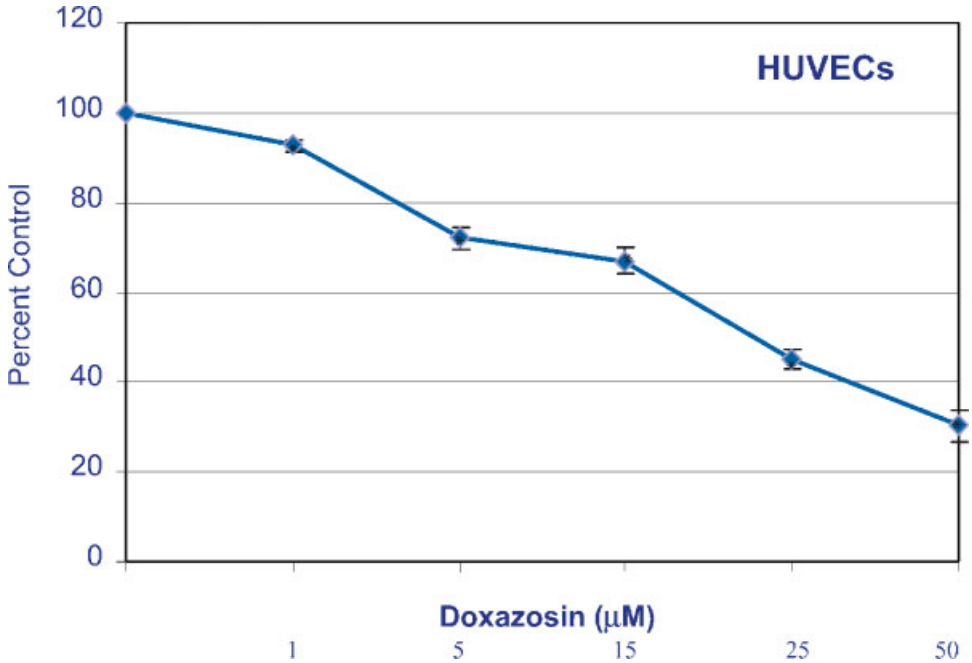

Fig. 1. Effect of doxazosin on cell viability of HUVEC cells. Subconfluent cultures of HUVEC cells were exposed to increasing concentrations of doxazosin (1–50 μM) and cell death was determined using the trypan blue exclusion assay. Values represent the mean percentage of cell viability from three independent experiments (performed in duplicate). (*) Asterisk indicates significant difference compared with the control at $0 < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Brugge, 1995]. We, therefore, examined the effect of doxazosin on endothelial cell attachment to fibronectin. HUVEC cells were plated on a fibronectin-coated surface and the effect of doxazosin (15 μM) or VEGF (15 ng/ml) or the combination treatment (doxazosin in the presence of VEGF) was evaluated after 24 h. Our data indicate that VEGF-mediated endothelial cell adhesion to fibronectin is markedly suppressed by doxazosin (Fig. 3-I and 3-IIA). In contrast, neither of the other two angiogenesis regulators, FGF-2 nor TGF-β exerted a significant effect on endothelial cell adhesion (Fig. 3-II, panels B and C, respectively). A negative effect by doxazosin against cell adhesion was also observed in human bone marrow (HBME) cells [Lehr and Pienta, 1998] (data not shown).

We subsequently determined the effect of doxazosin on endothelial cell migration using the wounding method. Figure 4 illustrates that at 24 h post-wounding of HUVEC cells, the control untreated cells migrated and almost filled the wounded area (Fig. 4-I, panels A and B, respectively). While there was an enhanced cell migration in the presence of exogenous VEGF

TABLE I. Effect of Doxazosin on Vascular Endothelial Cell Apoptosis

Treatment	% Apoptotic cells (24 h)	% Apoptotic cells (48 h)
Control	2.5 ± 1.0%	2.3 ± 0.8%
Doxazosin (5 μM)	4.8 ± 0.6%	16.1 ± 1.1%
Doxazosin (15 μM)	15 ± 2.1%	37.1 ± 2.9%

Following treatment with doxazosin, cells were stained with Hoechst 33342 and apoptotic cells were visualized and counted described in "Materials and Methods." Values represent the mean of the three independent experiments performed in duplicate (±SEM); $P < 0.01$.

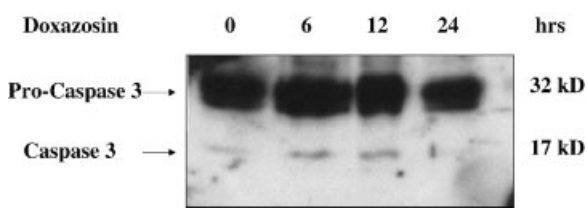

Fig. 2. Effect of doxazosin on caspase-3 activation in HUVEC cells. Cells were treated with doxazosin (25 μM) for various periods as indicated and cell lysates (30 μg samples of total protein) were subjected to electrophoretic analysis through 15% SDS–PAGE and Western blotting as described in ''Materials and Methods.'' The pro-caspase-3 (33 kDa) is detected in all samples while the proteolytic fragment indicating caspase-3 activation is detected after 6 h of doxazosin treatment (17 kDa).

(15 ng/ml) (Fig. 4-I, panel C), doxazosin treatment (15 μM) resulted in a dramatic decrease in HUVEC cell migration in the absence or presence of exogenous VEGF (Fig. 4D and 4E, respectively). Thus, doxazosin can also cause a significant suppression of VEGF-mediated cell migration.

To examine whether doxazosin can directly inhibit angiogenesis, the effect of doxazosin on VEGF-mediated tube formation by endothelial cells was investigated using an in vitro angiogenesis assay. Treatment with doxazosin (15 μM) severely suppressed angiogenesis induction (on the basis of tube formation) by $VEGF_{165}$ (20 ng/ml), while in the presence VEGF tube formation was strongly manifested (Fig. 5). Quantitative analysis of the data from this in vitro angiogenesis assay (the average number tubes for each treatment) is shown in Figure 5-II. The results indicate that the basal formation of capillary-like structures was stimulated by exogenous $VEGF_{165}$ (20 ng/ml). In the presence of doxazosin there was a significant inhibition of VEGF-induced tube number (Fig. 5-II), whereas an elevated tube number was detected in VEGF stimulated areas (Fig. 5-I).

Angiogenesis inhibitors can induce-tumor cell apoptosis by decreasing levels of endothelial-cell derived paracrine factors that promote cell survival [Meredith and Schwartz, 1997].

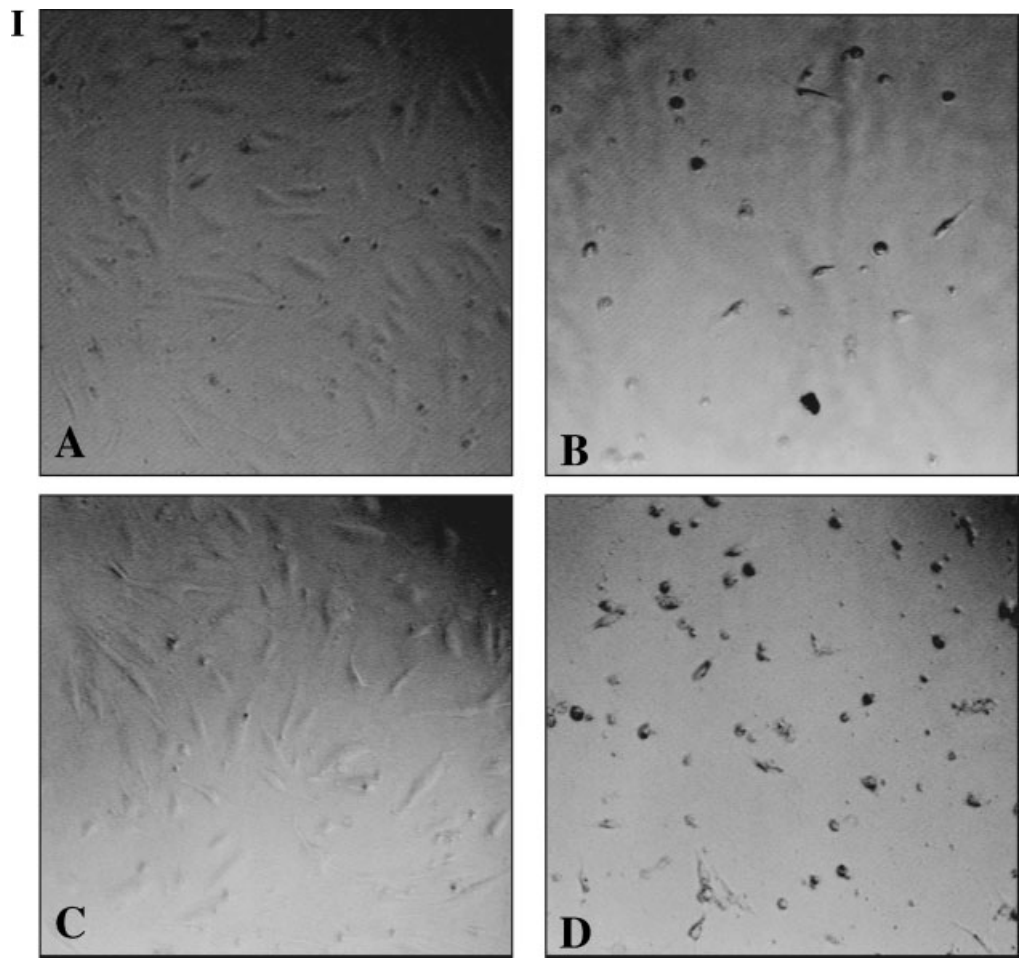

Fig. 3. **I**: Endothelial cell attachment to fibronectin-coated matrix. HUVEC cells were exposed for 24 h to normal growth media as a control (**A**), in the presence of doxazosin (15 μM) (**B**); in the presence of VEGF alone (15 ng/ml) (**C**); or in the presence of combined treatment (doxazosin and VEGF) (**D**), on fibronectin-coated plates. Cell attachment was determined as described in ''Materials and Methods.'' **II**: Effect of doxazosin on endothelial cell adhesion in presence of key cytokines, VEGF, FGF-2, and TGF-β. Quantitative evaluation of the data from the cell attachment assays revealed that doxazosin (15 μM) significantly inhibits endothelial cell attachment and it also antagonizes the stimulating effect of VEGF on cell adhesion to fibronectin-matrix ($P < 0.01$) (**panel A**). There was no significant effect on FGF-2 mediated endothelial cell attachment (**panel B**), or TGF-β (**panel C**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

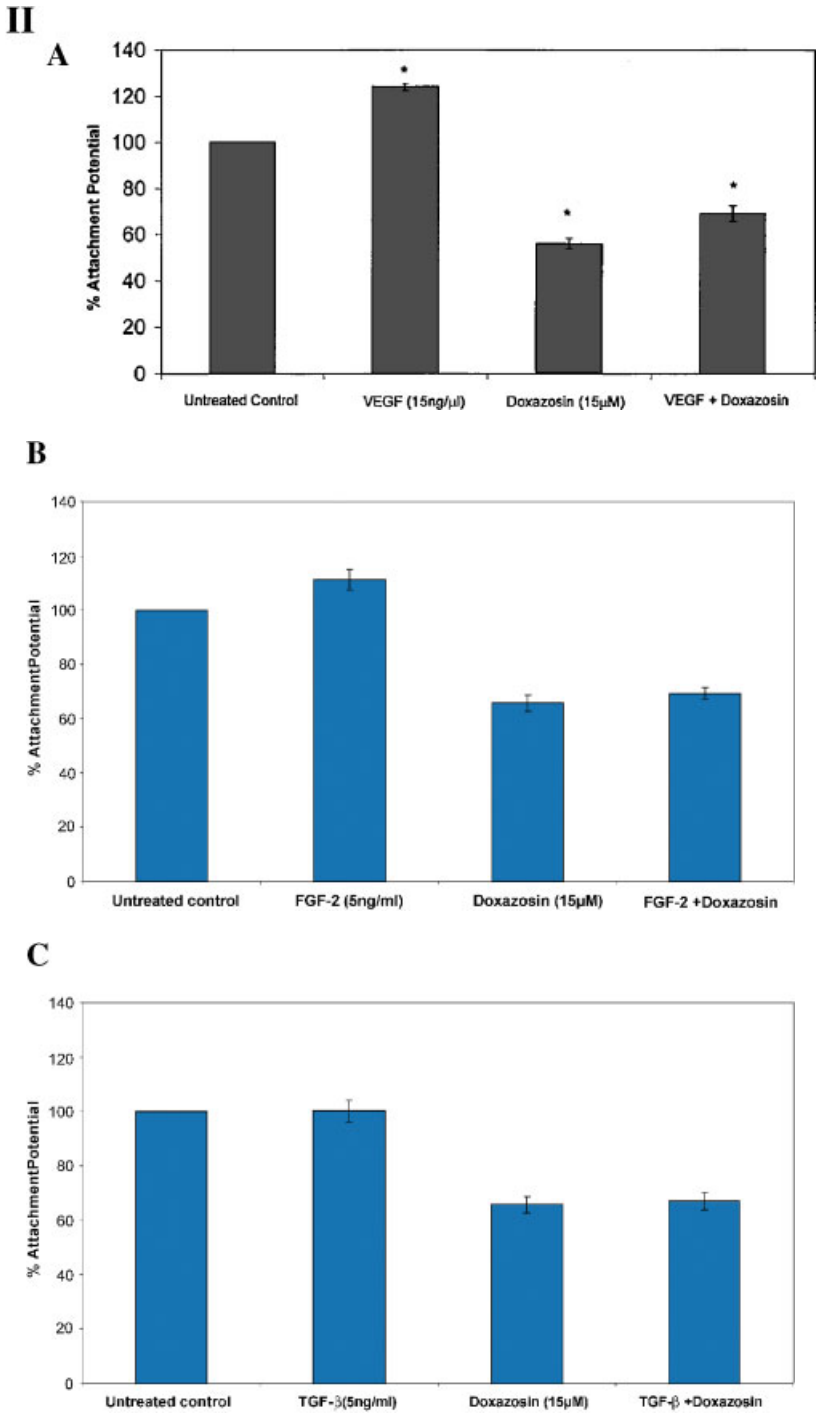

Fig. 3. (*Continued*)

To determine the cytokine specificity in terms of doxazosin's ability to target VEGF during inhibition of stimulated HUVEC cell invasion and tube formation, the effect of another potent angiogenic factor FGF-2 was subsequently investigated. The results shown on Figure 6 indicate that FGF-2 results in a significant stimulation of HUVEC angiogenic response that was suppressed by doxazosin treatment ($P < 0.05$). TGF-β on the other hand, had no significant impact on HUVEC-tube formation (Fig. 6-II, panel B).

To gain a mechanistic insight into the negative effect of doxazosin against endothelial

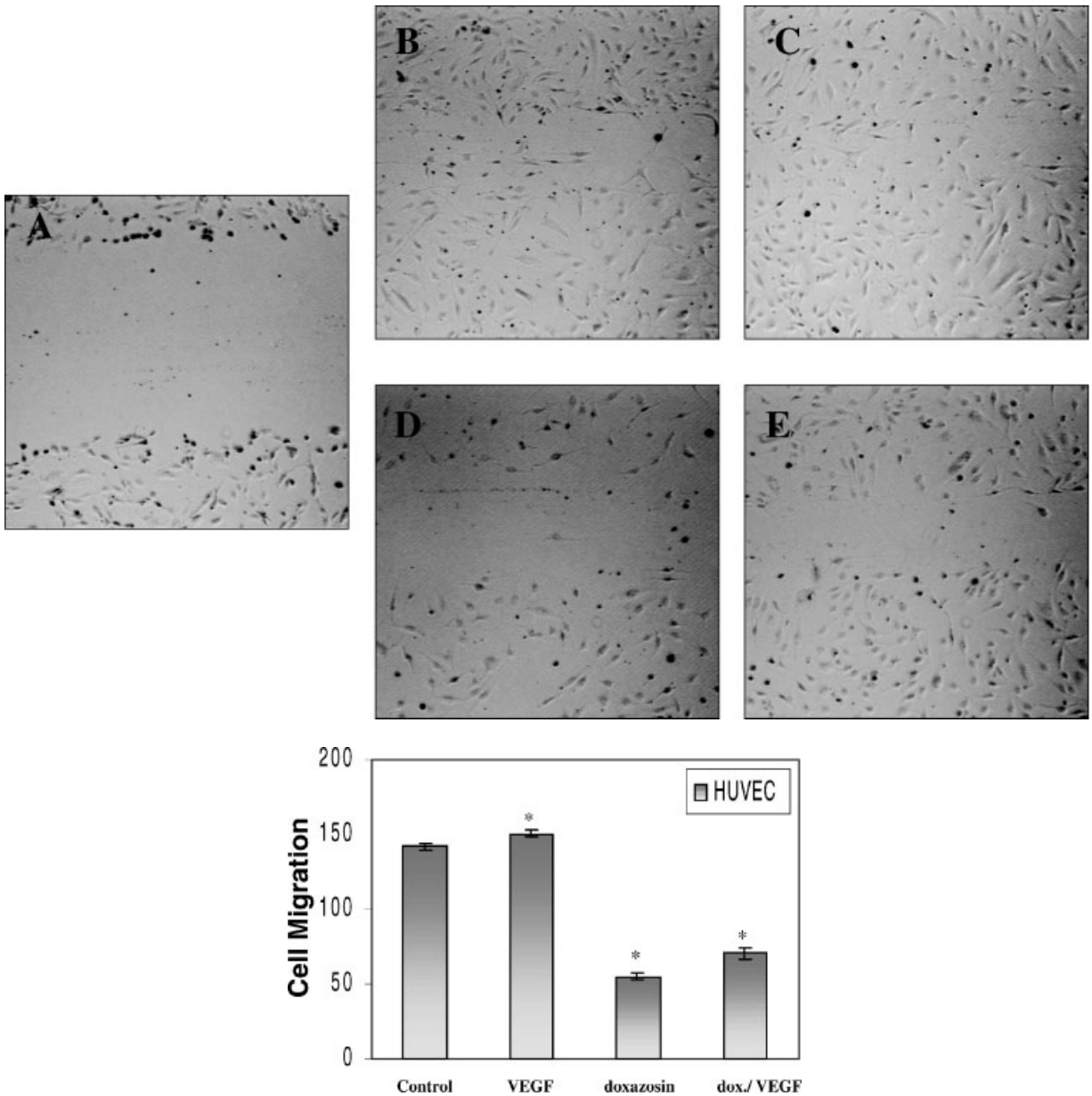

Fig. 4. **I**: Assessment of endothelial cell migration using the wounding assay. After cell-wounding was induced as described in ''Materials and Methods,'' cells were washed and subsequently exposed to doxazosin alone, VEGF alone or the combination treatment of doxazosin in the presence of VEGF. At wounding, (**A**); after 24 h, vehicle control (**B**); in the presence of VEGF (15 ng/ml) (**C**); in the presence of doxazosin (15 μM) (**D**); in the combined presence of VEGF (15 ng/ml), and doxazosin (15 μM) (**E**). **II**: Quantitative analysis of doxazosin's effect on HUVEC cell migration. The number of cells in the wounded area were counted after various treatments as shown and values were expressed as the percentage of the values for the untreated (vehicle) controls. Values represent mean of two different fields from three independent experiments ± S.E.M. Asterisk indicates statistical significance at $P < 0.01$.

cell adhesion, migration, and angiogenesis we examined the expression profiles of VEGF and its receptor KDR in HUVEC cells. The RT-PCR analysis shown on Figure 7 revealed that doxazosin treatment for 24 h resulted in a significant downregulation of VEGF mRNA (for all three isoforms) but had no effect on KDR expression levels. To determine whether VEGF synthesis might also be regulated at a post-transcriptional level, we examined the levels of VEGF secreted in the medium of doxazosin treated-HUVEC cells, using specific ELISA assays. The results summarized on Table II indicate no significant changes in VEGF protein levels

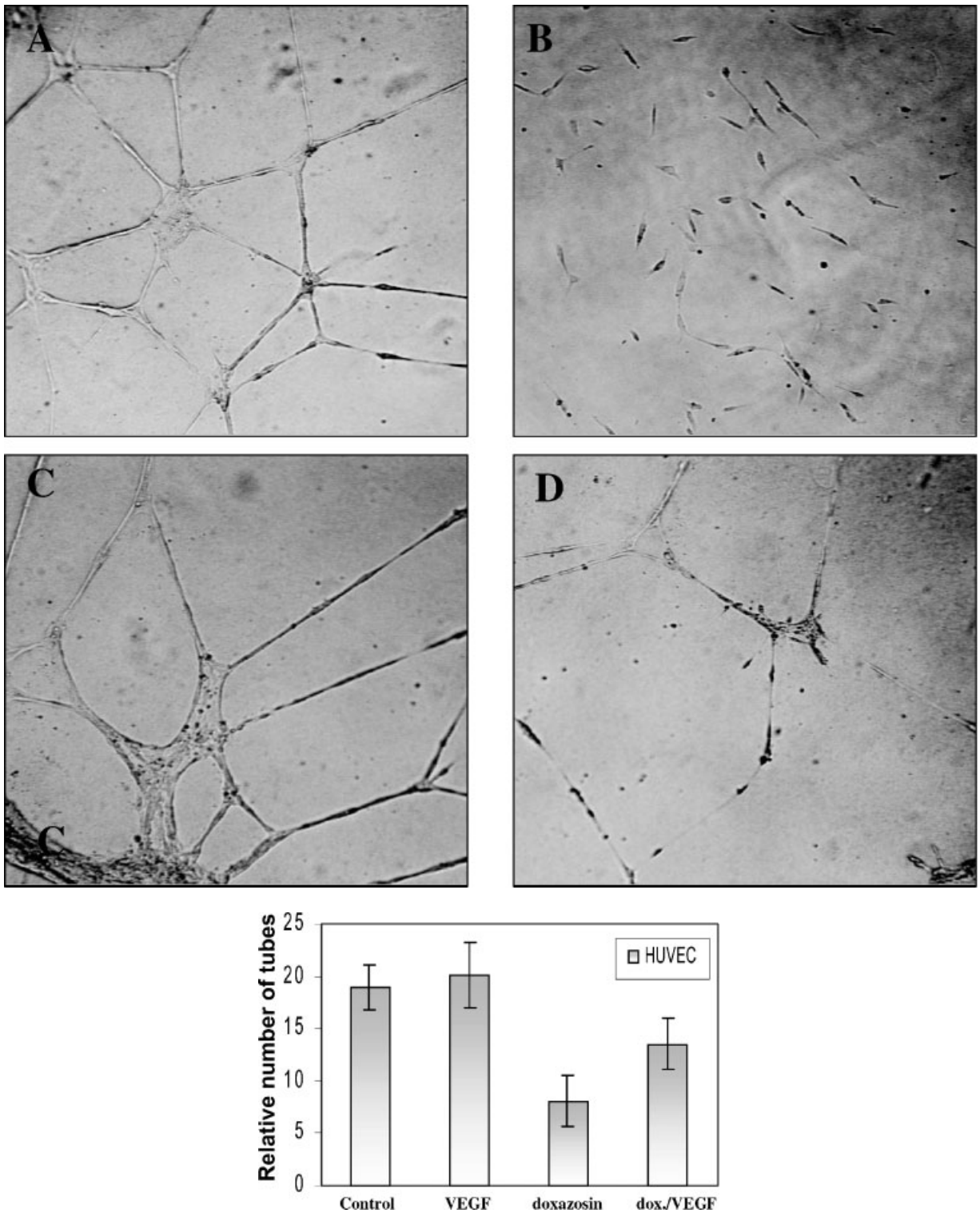

Fig. 5. **I**: Effect of doxazosin on HUVEC in vitro angiogenesis. The angiogenic response of human endothelial cells to VEGF was evaluated in the presence or absence of doxazosin by examining the tube formation in HUVECs on Matrigel (in vitro basement membrane). HUVEC (10×10^4 cells/well) were seeded in 96-well plates onto ECMatrigel after 24 h in vitro angiogenesis was assessed as formation of capillary-like structures of HUVEC, as described in ''Materials and Methods.'' **Panel A**: Vehicle control; (**panel B**) treatment with doxazosin (15 μM); (**panel C**) in the presence of VEGF (15 ng/ml) alone; and (**panel D**) in the presence of combination treatment of VEGF (15 ng/ml) and doxazosin (15 μM). **II**: Quantitative evaluation of VEGF-mediated in vitro tube formation and the impact of doxazosin. The number of capillary-like tubes was counted in each well using light microscopy imaging (Axiovert 10, Zeiss) as described in ''Materials and Methods.'' Results represent the mean values form three independent experiments (performed in duplicate) ±S.E.M.

secreted by endothelial cells throughout the 72-h treatment period with doxazosin. There was however a significant elevation in FGF-2 protein levels after 72 h of exposure to doxazosin compared to the untreated control cells ($P < 0.05$) (Table II).

Analysis of the impact of doxazosin on FAK protein levels in endothelial cells demonstrated a moderate decrease of FAK protein expression (20%) after 12 h of exposure to the drug compared to the untreated control cells (Fig. 8).

DISCUSSION

Tumors can activate the angiogenic switch by changing the balance of angiogenesis inducers and inhibitors and the angiogenesis regulators expressed by human tumors offer attractive therapeutic targets. Driven by the recently documented anti-angiogenic and apoptotic action of the quinazlone-based α1-adrenoceptor antagonists (doxazosin and terazosin) against prostate benign and malignant epithelial cells [Kyprianou and Benning, 2000; Keledjian et al.,

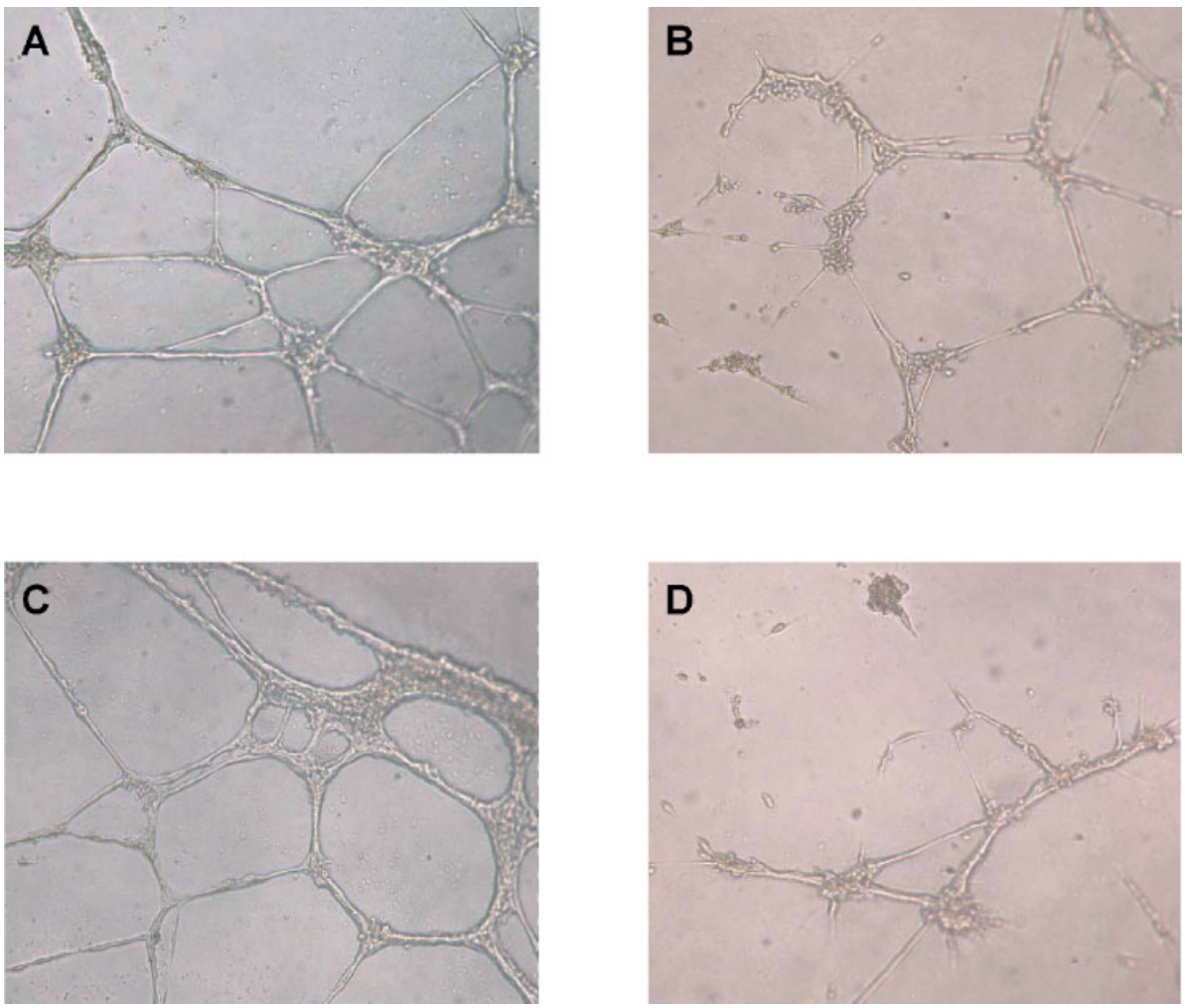

Fig. 6. **I**: The angiogenic response of human endothelial cells to FGF-2 was evaluated in the presence or absence of doxazosin by examining the tube formation in HUVECs on Matrigel as described in the legend for Figure 5-I. In vitro angiogenesis was assessed as formation of capillary-like structures of HUVEC cells after 24 h of incubation with doxazosin. **Panel A**: Vehicle control; (**panel B**) treatment with doxazosin (15 μM); (**panel C**) in the presence of FGF-2 (5 ng/ml) alone; and (**panel D**) combined presence of FGF-2 (5 ng/ml) and doxazosin (15 μM). **II**: Impact of doxazosin on FGF-2 and TGF-β mediated human vascular endothelial cell invasion and tube formation. **A**: Doxazosin inhibits FGF-2 mediated HUVEC-angiogenic behavior. **B**: Lack of TGF-β effect on HUVEC-tube formation. Data shown represent the mean values from three independent experiments (performed in duplicate) ±SEM. *, denotes statistically significant difference from untreated control; **, denotes statistically significant difference from doxazosin-treated HUVECs. $P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

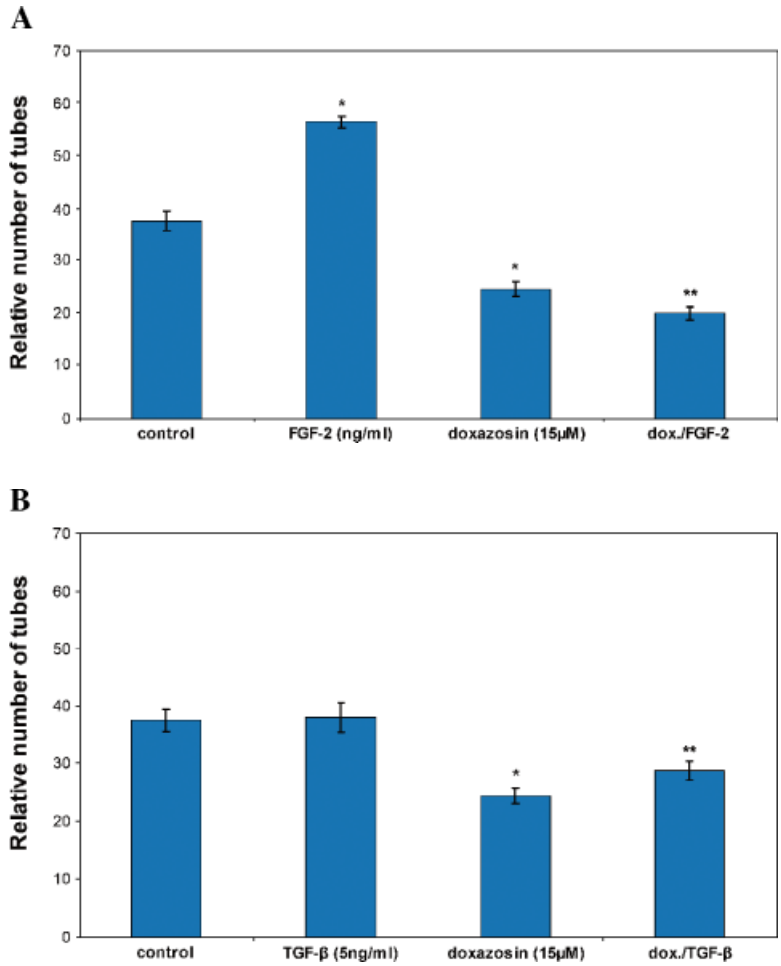

Fig. 6. (*Continued*)

2001; Benning and Kyprianou, 2002], in this study we examined the direct effect of doxazosin on human vascular endothelial cell death, migration, and tube formation.

Considerable information exists concerning the role of α1-adrenoceptor antagonists in organs other than the prostate such as the heart and vascular smooth muscle. The quinazoline-derived α1-adrenoceptor antagonists, doxazosin and prazosin, have been shown to inhibit vascular injury-induced hyperplasia of arterial vascular smooth muscle cells (VSMC) [O'Malley et al., 1989; Fingerle et al., 1991] an action with functional significance in vivo in preventing intimal hyperplasia in rat and rabbit models of vascular injury [O'Malley et al., 1989; Vashisht et al., 1992]. The present findings provide the first evidence on the ability of doxazosin (a quinazoline-derived α1-adrenoceptor antagonist) to induce apoptosis, suppress migration and invasion and inhibit the angiogenic response of human vascular endothelial cells to VEGF. Direct support for our observations stems from a recent independent report demonstrating the ability of terazosin, another α1-adrenoceptor antagonist, to exert antiangiogenic and apoptotic action against human prostate tumor epithelial and endothelial cells [Pan et al., 2003]. Moreover there is evidence indicating that doxazosin is a potent inhibitor of VSMC proliferation and migration through a mechanism unrelated to α1-adrenoceptor antagonism [Hu et al., 1998], an it also inhibits chemotaxis-directed migration of human monocytes [Kintscher et al., 2001] and proliferation of human coronary artery smooth muscle cells by blocking cell cycle progression [Kintscher et al., 2000]. While similar doxazosin-directed apoptotic effect was observed for bone marrow endothelial cells, the specificity/significance of the

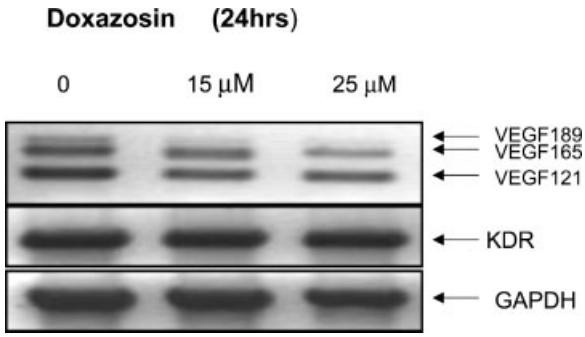

Fig. 7. RT-PCR analysis of VEGF expression and its receptor in response to doxazosin treatment of endothelial cells. HUVEC cells were treated with increasing concentrations of doxazosin and RNA was isolated and subjected to RT-PCR analysis as described in ''Materials and Methods.'' Doxazosin reduced the expression of all three isoforms of VEGF ($VEGF_{189}$, $VEGF_{165}$, and $VEGF_{121}$), but not the receptor KDR in a dose-dependent manner. GAPDH was analyzed as a normalizing control.

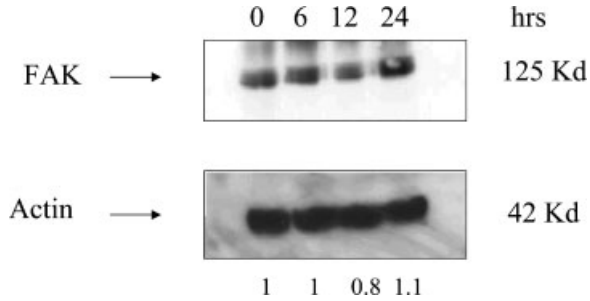

Fig. 8. Western blot analysis of focal adhesion kinase (FAK) expression in HUVEC cells after doxazosin treatment. Cells were treated with doxazosin (25 μM) 6, 12, and 24 h. Protein extracts from control and doxazosin-treated HUVEC cells were immunoblotted with FAK antibody as described in ''Materials and Methods.'' Actin expression was used as a normalizing control. Molecular weights are shown on the right.

angiogenic effect of doxazosin in prostate-tumor derived endothelial cells remains to be defined.

In addition to promoting endothelial cell proliferation and invasion, VEGF can protect endothelial cells from inducers of apoptosis [Ferrara and Davis-Smyth, 1997]. The in vitro response to doxazosin of human endothelial cells involved temporal activation of the apoptotic machinery within 6 h of treatment (caspase-3 activation), that precedes extensive cell death, and suppression of cell invasion and angiogenic growth (after 24 h). Significantly enough doxazosin induces human endothelial cell death by activating apoptosis at concentrations lower than those previously shown to induce apoptosis of prostate cancer cells [Benning and Kyprianou, 2002]. The temporal pattern and the kinetics of the anti-growth effect in HUVEC cells indicate a higher sensitivity of vascular endothelial cells to doxazosin-induced apoptosis compared to prostate tumor epithelial cells [Kyprianou and Benning, 2000], and resonate with data from an experimental prostate tumor system, in which endothelial cell apoptosis preceded tumor-cell apoptosis by 3–4 days [Browder et al., 2000]. Studies are currently in progress to determine the in vivo antiangiogenesis efficacy in an experimental tumor angiogenesis model; it is nevertheless tempting (while waiting for the outcome), to propose that the antiangiogenic effect observed here provides an attractive molecular basis for the suppression of human prostate tumor vascularity by the other quinazoline, terazosin in BPH patients [Keledjian et al., 2001].

TABLE II. Effect of Doxazosin (15 μM) on Vascular Endothelial Growth (VEGF) and Basic Fibroblast Growth Factor (FGF-2) Secretion in HUVEC Cells

Time (h)	VEGF (ng/ml)	FGF-2 (ng/ml)
0	0.83 ± 0.08	0.46 ± 0.06
6	0.94 ± 0.01	0.65 ± 0.03
12	0.93 ± 0.02	0.47 ± 0.08
24	1.02 ± 0.02	0.68 ± 0.12
48	0.97 ± 0.02	0.61 ± 0.07
72	0.94 ± 0.01	0.76 ± 0.03[a]

Values represent the mean ± SEM (from two independent experiments performed in duplicate).
[a]Statistical significance at $P \leq 0.05$.

The observed antiangiogenic effect of doxazosin might not be unique to the VEGF migration-invasion signaling axis, but additional angiogenic factors such as FGF-2 are potentially targeted as demonstrated by the inhibitory effect of doxazosin on both VEGF and FGF-2-mediated vascular endothelial invasion and tube formation. FGF-2 however had no significant effect on endothelial cell adhesion. Taken together these data support an action by doxazosin as a general anti-angiogenic agent and while there is a heavy involvement of VEGF at the level of adhesion, migration and invasion of endothelial cells, the targeting effect at the tube formation level (as a final angiogenic step) might not be entirely unique to the VEGF adhesion axis, with the recruitment of FGF-2.

Suprisingly, the present data do not support a mechanistic link with the TGF-β signaling in the execution of doxzazosin's inhibitory effect on adhesion and invasion of vascular endothelial cells. This is somewhat surprising considering the recent data from our laboratory

indicating TGF-β activation as an underlying mechanism of doxazosin-induced apoptosis of human prostate tumor epithelial cells [Partin et al., 2003]. The differential response might reflect the specificity of the cellular response to the drug (antiangiogenesis vs. apoptosis induction), and cell type (endothelial vs. epithelial cells).

A downregulation of VEGF (but not its receptor) has been detected in this study as an initial event triggered by doxazosin in HUVEC cells. VEGF-induced angiogenesis depends on integrins and the blockade of endothelial integrin αvβ3 inhibit angiogenesis [Carmeliet et al., 1999; Byzova et al., 2000]. In view of the number of intracellular effectors of VEGF towards the execution of angiogenic response, such as phosphorylation of FAK, MAPK, phosphatidylinositol 3-kinase, and paxillin in HUVEC cells [Guo et al., 1995; Schlaepfer and Hunter, 1998], the possibility emerges that doxazosin may interfere with the post-translational modification of these key VEGF-targeted molecules in inhibiting the angiogenic response of endothelial cells. The events involved in tumor progression toward metastasis are largely mediated by the integrins which upon engagement with components of the ECM, reorganize to form focal adhesion complexes [Clark and Brugge, 1995]. The present findings indicate that during doxazosin-mediated apoptosis in endothelial cells there is a reduced FAK protein expression. This might be of mechanistic value considering the significance of focal adhesion kinase in the control of adhesion-dependent survival of epithelial and endothelial cells [Frisch et al., 1996] and the involvement of focal adhesion signaling in the migration of prostate cancer cells [Zheng et al., 1999]. Indeed, given the established role for FAK in the regulation of integrin signaling, the proteolytic cleavage of FAK by caspase-3 reported during growth factor deprivation-induced apoptosis in HUVEC cells [Levkau et al., 1998] implicates FAK as a "high-profile" intracellular signaling effector that might be targeted by the quinazolines. Support for this concept stems from recent evidence indicating deregulation of FAK expression during doxazosin-induced apoptosis of human prostate cancer cells [Walden et al., 2002].

In addition to FAK, other intracellular effectors of the integrin pathway such as akt and catenin, key elements of anchorage-mediated survival signaling of cancer cells [Lauffenburger and Horwitz, 1996; Acollo and Pepper, 1999], might be functionally involved in directing doxazosin's angiogenic action. Collagen type IV has been shown to be proteolytically cleaved into peptide fragments including tumstatin, canstatin, and arresten that act as angiogenesis inhibitors by inhibiting endothelial cell proliferation, inducing apoptosis, and suppressing tumor growth, respectively [Colorado et al., 2000; Maeshima et al., 2002]. As the present results suggest that doxazosin might exert all three functions against endothelial cell growth, one could easily argue that the quinazoline-based α1-adrenoceptor antagonists target the angiogenic capability of both vascular endothelial and prostate epithelial cells, an activity that is not shared by the sulphonamide-based antagonist, tamsulosin [Keledjian and Kyprianou, 2003].

In summary, the present results may be relevant to the therapeutic targeting of angiogenesis associated with tumor growth, where a pro-survival signaling primarily mediated by VEGF may alter the threshold level of endothelial cell susceptibility to doxazosin's apoptotic effects. Since tumor angiogenesis correlates with metastasis in invasive prostate carcinoma [Weidner et al., 1993], identification of the signaling pathways targeted by the quinazolines in endothelial cells will enable the development of effective combination approaches for disruption of tumor-associated angiogenesis in metastatic prostate cancer.

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