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Corticosteroids inhibit the expression of the vascular endothelial growth factor gene in human vascular smooth muscle cells

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Abstract

The vascular endothelial growth factor (VEGF) is a specific mitogen for vascular endothelial cells and enhances vascular permeability and edemagenesis. VEGF is also a major regulator of angiogenesis and may be a key target for inhibiting angiogenesis in angiogenesis-associated diseases. Among the extensively studied angiostatic compounds are several corticosteroids when used alone or in combination with heparin. In this study we present evidence for an additional mechanism of action of hydrocortisone, cortisone and dexamethasone in inhibiting edemagenesis or angiogenesis. In cultures of aortic human vascular smooth muscle cells these corticosteroids (1×10^{-8} to 1×10^{-12} M) abolished the platelet-derived growth factor-induced (PDGF) expression of the VEGF gene in a dose-dependent manner. In contrast, two precursors of corticosteroids, desoxycorticosterone or pregnenolone, did not affect PDGF-induced VEGF expression. Our findings indicate that the capacity of corticosteroids to reduce edema or to prevent new blood vessel formation may be attributed, at least in part to the ability of these agents to abolish the expression of VEGF. © 1998 Elsevier Science B.V.

Keywords: Corticosteroid; Desoxycorticosterone; Pregnenolone; Anti-angiogenic; VEGF (vascular endothelial growth factor)

1. Introduction

The multifunctional cytokine vascular endothelial growth factor (VEGF) is a homodimeric, 34 to 46 kDa, heparin-binding, disulfide-bonded glycoprotein (Tischer et al., 1991). VEGF acts as a vascular endothelial cell-specific mitogen (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989), enhances vascular permeability (Keck et al., 1989; Leung et al., 1989), edemagenesis (Criscuolo, 1993) and it promotes angiogenesis (Leung et al., 1989). VEGF is expressed and secreted by numerous tumor and normal tissue cell lines of animal and human origin (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989; Harada et al., 1994; Koch et al., 1994; Pertovaara et al., 1994; Shifren et al., 1994; Frank et al., 1995; Wizigmann-Voos et al., 1995) including human vascular smooth muscle cells (Tischer et al., 1991; Neufeld et al., 1994). Of the four different human isoforms of VEGF only $VEGF_{121}$ and the heparin-dependent $VEGF_{165}$ express mitogenic and angiogenic activity (Gengrinovitch et al., 1995). VEGF is inhibited by α_2-macroglobulin (Soker et al., 1993), while hypoxia (Brogi et al., 1994; Levy et al., 1995) and hypoglycemia (Stein et al., 1995) are potent stimuli for VEGF gene expression.

The tyrosine-kinase receptors encoded by the flt and KDR/flk-1 genes were found to function as the VEGF receptors. Both receptors have been reported to be exclusively expressed on the cell surface of vascular endothelium (De Vries et al., 1992; Millauer et al., 1994; Neufeld et al., 1994; Flamme et al., 1995). However, recent reports indicate that specialized cells such as uterine smooth muscle cells (Brown et al., 1997) may express VEGF receptors.

The fact that VEGF has not been localized in endothelial cells indicates a central paracrine mechanism of action. VEGF is secreted by non endothelial cells under the

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influence of other cytokines such as transforming growth factor-β (Pertovaara et al., 1994) or platelet-derived growth factor (PDGF) (Brogi et al., 1994), and modulates activities in adjacent vascular endothelium (Shifren et al., 1994). Thus, VEGF appears to be a major regulator for inducing angiogenesis and may be proved to be a molecule of potential therapeutic value. In this respect, VEGF produced significant augmentation of collateral vessel development in experimentally induced unilateral hind limb ischemia, indicating that the angiogenic activity of VEGF may be sufficiently potent to achieve therapeutic benefit to patients with severe limb ischemia secondary to arterial occlusive disease (Takeshita et al., 1994).

VEGF may also be a key target for inhibiting angiogenesis, a process which today is considered a valid therapeutic strategy for an array of angiogenesis-associated diseases (Maragoudakis et al., 1992; Auerbach and Auerbach, 1994; Folkman, 1995), ever since Folkman pioneered the concept that inhibition of angiogenesis might represent a suitable complementary strategy for the treatment of solid tumors (Folkman, 1972). Among some of the extensively studied inhibitors of angiogenesis are the corticosteroids: medroxyprogesterone, dexamethasone, triamcinolone and cortisone and it has been proposed that the first two agents may act by inhibiting plasminogen activator or basic fibroblast growth factor (reviewed by Auerbach and Auerbach, 1994). Cortisone and hydrocortisone have also been reported to inhibit angiogenesis but only when used in combination with heparin (Folkman et al., 1983; Folkman and Ingber, 1987; Maragoudakis et al., 1989; reviewed by Auerbach and Auerbach, 1994). The anti-angiogenic effect of heparin in combination with hydrocortisone or cortisone, by an as yet unknown mechanism of action, was shown to be specific for growing capillaries, whereas mature non-growing capillaries remain unaffected (Folkman, 1995).

In this study we have shown that cortisone, hydrocortisone and dexamethasone, but not their precursors desoxycorticosterone or pregnenolone, can inhibit the messenger RNA (mRNA) expression of VEGF in human vascular smooth muscle cells obtained from the pulmonary aorta. Our findings reveal an additional mechanism to explain the ability of these agents to reduce edema and indicate that their anti-angiogenic action, when used alone or in combination with heparin, may be attributed, at least in part, to the ability of these corticosteroids to abolish the transcription of VEGF mRNA.

2. Methods

2.1. Generation and characterization of primary human vascular smooth muscle cells

Ten cell lines of vascular smooth muscle cells were established from pulmonary arteries obtained from patients undergoing surgical treatment. Arteries were kept overnight in Hank's buffered salt solution (Seromed, Fakola, Basel, Switzerland) at 4°C before stripping off the media intima by forceps. The vessels were cut into small pieces (3–5 mm). These pieces of arterial tissue were transferred into a cell culture flask (Falcon), pre-wetted with culture medium. Cells were generated by incubating the vessel tissue for one week in Dulbecco's minimal essential medium (Seromed, Fakola) supplemented with 5% fetal calf serum (Gibco/BRL, Basel, Switzerland) and 20 mM HEPES (Seromed, Fakola). Cells were cultivated in the same medium and subcultures were set up after trypsin treatment.

Subcultured vascular smooth muscle cells were characterized and their authenticity was ensured using a monoclonal antibody specific for smooth muscle cells (Boehringer Mannheim, Mannheim, FRG), as previously described (Roth et al., 1991, 1996). In brief, cells were grown in Lab-Tek tissue culture chamber/slides (Miles, Scientific Div., Naperville, IL) until confluence and fixed in 4% paraformaldehyde. Non-specific protein binding was blocked by incubating the cells in phosphate buffered saline containing 0.5% (w/v) bovine serum albumin (Fluka, Buchs, Switzerland) for 20 min, followed by incubation with the first mouse anti-smooth muscle cell actin specific antibody (60 min), washing with phosphate buffered saline ($3\times$) and further incubation with fluorescein isothiocyanate-coupled rabbit anti-mouse IgG (Boehringer). Preparations, were then washed with phosphate buffered saline ($3\times$), mounted with Fluorosave reagent (Calbiochem-Novabiochem, Lucerne, Switzerland) and observed on a microscope equipped with epillumination and specific filters (560 nm) (Axiophot; Carl Zeiss, Wetzlar, FRG) as described earlier (Roth et al., 1991, 1996).

Subconfluent cultures of vascular smooth muscle cells were serum-deprived for 48 h with low serum medium (Dulbecco's minimal essential medium supplemented with 0.5% fetal calf serum and 20 mM HEPES) before being stimulated with either one of the three PDGF isoforms (PDGF-AA, -AB or -BB; Gibco/BRL), in the presence or absence of various concentrations (1×10^{-8} to 1×10^{-12} M) of either cortisone, hydrocortisone, dexamethasone (Sigma, Buchs, Switzerland), or one of the cholesterol precursors of the corticosteroids: desoxycorticosterone or pregnenolone (Sigma). Cells stimulated with vehicle (0.001% ethanol in water) alone were used as control. The period of stimulation with each mitogen was 0 to 12 h in kinetic studies for the transcription assays and 0–48 h for secretion of VEGF protein. Medium was exchanged every 12 h to avoid autostimulation of the cells. All experiments were performed using cell cultures during passages 2 to 5.

2.2. Isolation of RNA and Northern blotting

Transcripts encoding for VEGF or for the constitutive HLA-β genes was determined using Northern-blot analysis

(Roth et al., 1991, 1996). RNA was extracted at various time points (0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h). Total RNA was extracted as described by Chomczynski and Sacchi (1987) and 10 μg of total RNA were size fractionated in a standard 1% agarose gel containing morpholino-sulphonate and 2% formaldehyde (Roth et al., 1991, 1996). RNAs were transferred onto Hybond nylon membranes (Amersham, Little Chalfort, UK) by capillary blotting for overnight in a 10-fold concentration of saline–sodium citrate buffer (1-fold saline–sodium citrate buffer = 1.5 mM NaCl, 15 mM Na_3–citrate, pH 7.0) and cross linked by ultraviolet irradiation. Membranes were prehybridized for 1 h at 45°C with QuickHyb (Stratagene, Heidelberg, FRG) supplemented with 10 μg/ml heat denatured salmon sperm DNA. Blots were either hybridized overnight with 20 ng of 3′-end-labeled ([α^{32}P]dATP) VEGF-antisense oligonucleotide probes (R&D, Abingdon, UK) or with a random prime labeled human leucocyte antigen (HLA)-β cDNA probe (ATCC 57474) (American Type Culture Collection), used as constitutive control (Roth et al., 1991, 1996). Blots were washed as follows: twice (15 min each) with a 5-fold concentration of saline–sodium citrate buffer, at room temperature and finally with 0.1-fold concentration of the same buffer, at 55°C for the VEGF probe and at 68°C for the HLA-β probe, respectively. Blots were exposed to Kodak X-Omat films overnight or up to 48 h at −70°C. Hybridization signals were densitometrically analyzed using an automated scanner system and the NIH-image program from Macintosh.

2.3. Expression of VEGF protein

Protein of VEGF was analyzed using enzyme linked immunosorbent assays (EIA, R&D). In brief, cells (1×10^4 per ml) were seeded onto a 12 well cell culture plate (Falcon) and cultivated until they achieved 80% confluence. The cells were brought to quiescence by cultivation for 48 h with Dulbecco's minimal essential medium supplemented with 0.5% fetal calf serum and 20 mM HEPES, followed by stimulation with one of the PDGF-isoforms (10 ng/ml), in the presence or absence of one of the corticosteroids or their precursors (1×10^{-8} to 1×10^{-12} M). Aliquots (500 μl) of the cell culture medium were collected at various time points (0, 12, 18, 24, 36 and 48 h) and the content of secreted VEGF was determined for each sample in duplicate using a commercially available VEGF-EIA kit.

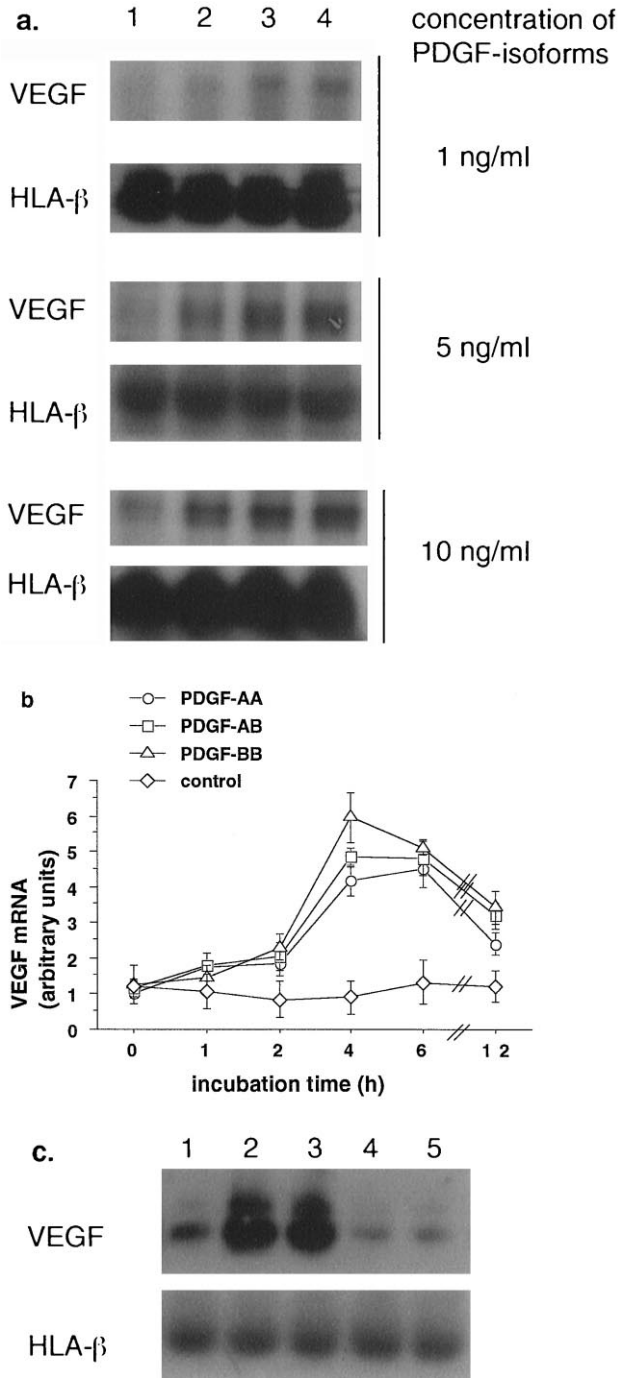


Fig. 1. Northern-blot analysis of the effects of PDGF-isoforms on the expression of the VEGF gene in cultivated human vascular smooth muscle cells. (a) Representative Northern-blot of the effect of various concentrations of PDGF-AA, -AB and -BB isoforms (1, 5 and 10 ng/ml) on the transcription of the VEGF and the HLA-β gene in human vascular smooth muscle cells: lane 1, unstimulated control; lane 2, PDGF-AA; lane 3, PDGF-AB and lane 4, PDGF-BB. Expression of mRNA was analysed 4 h after stimulation. (b) Kinetic study: effect of PDGF-isoform-induced transcription of the VEGF gene, at different time points after stimulation with each mitogen (5 ng/ml). The curves represent a typical pattern of mitogen-induced kinetics of VEGF gene activation from a single experiment. Similar results were obtained with five other vascular smooth muscle cells lines. (c) Effect of actinomycn D 10 μg/ml on PDGF-induced transcription of the VEGF gene at time point of maximal transcription (4 h after stimulation). Lane 1, unstimulated control; lane 2, PDGF-AA (10 ng/ml); lane 3, PDGF-BB (2.5 ng/ml); lane 4, PDGF-AA + actinomycin D (10 μg/ml) and lane 5, PDGF-BB + actinomycin D (10 μg/ml).

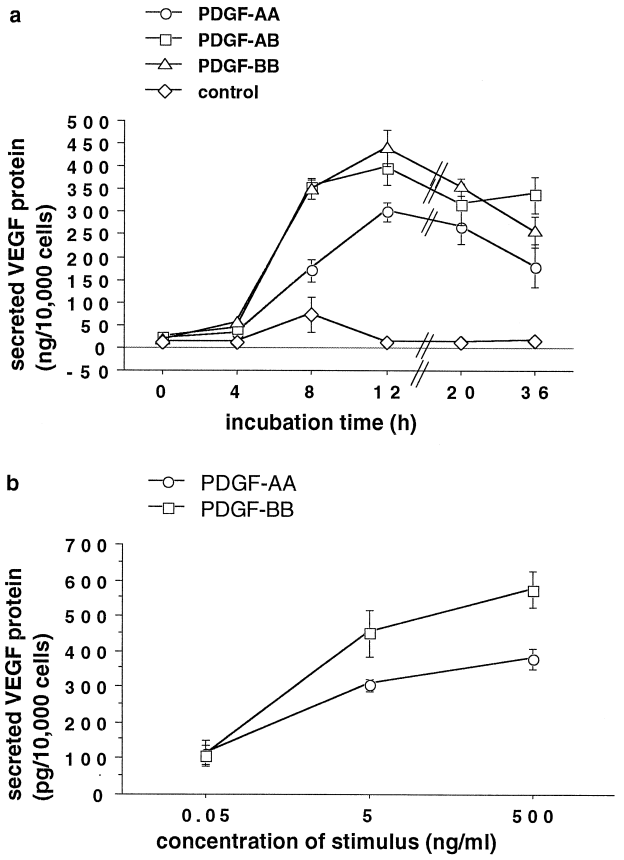


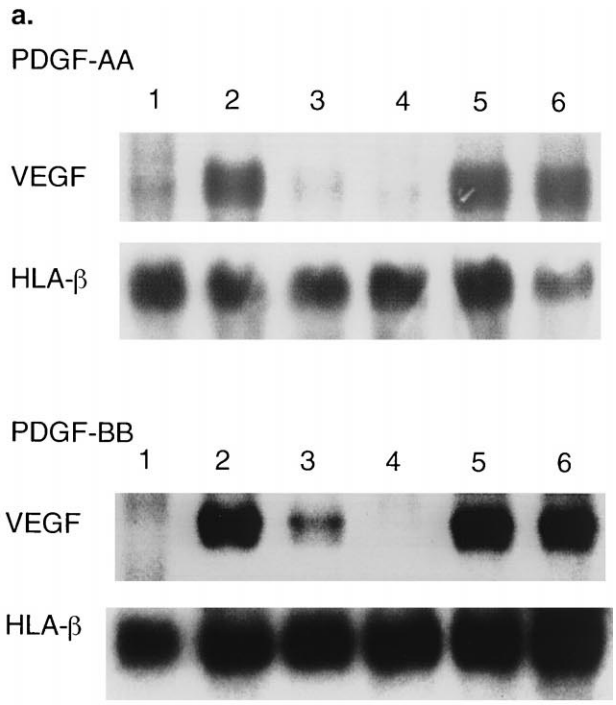
Fig. 2. (a) Kinetics of PDGF-induced secretion of VEGF into the culture medium of human vascular smooth muscle cells, determined by EIA. Concentrations of the three PDGF-isoforms correspond to their ED_{50} values, as provided by the distributor (PDGF-AA = 10 ng/ml; PDGF-AB = 5 ng/ml; PDGF-BB = 2.5 ng/ml). Control = cells stimulated with vehicle alone. (b) Dose-dependency of PDGF-AA and PDGF-BB induced secretion of VEGF in vascular smooth muscle cells. Each point represents the mean ± S.E. from six vascular smooth muscle cells lines.

To achieve correlation of VEGF protein secretion to cell numbers, cells were washed twice with phosphate buffered saline (PBS, w/o Ca^{2+}, Mg^{2+}, Seromed, Fakola) and harvested by trypsin/EDTA treatment. Cells were centrifuged ($600 \times g$, 15 min., 4°C) and cell pellets were resuspended in 1 ml of PBS and counted using a Neubauer chamber slide (Brand, Germany).

3. Results

3.1. PDGF-induced transcription of VEGF mRNA and secretion of VEGF in cultivated human vascular smooth muscle cells

All three PDGF-isoforms induced the transcription of VEGF mRNA (Fig. 1a) and secretion of VEGF (Fig. 2) in



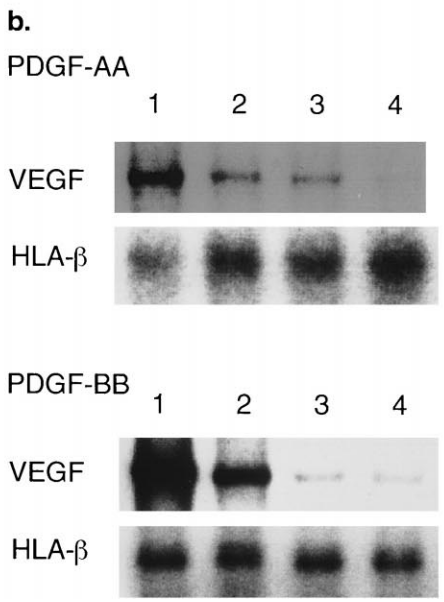


Fig. 3. Effect of corticosteroids on PDGF-induced expression of the VEGF gene and on secretion of VEGF protein in cultivated human vascular smooth muscle cells. (a) Representative Northern-blot of the effect of glucocorticoids and their precursors on PDGF-AA- or -BB-induced transcription of the VEGF gene evaluated 4 h after stimulation with one of the mitogens: lane 1, unstimulated control; lane 2, stimulus alone (PDGF-AA: 10 ng/ml, PDGF-BB: 2.5 ng/ml, respectively); lane 3, PDGF + cortisone (1×10^{-9} M); lane 4, PDGF + hydrocortisone (prednisolone: 1×10^{-9} M); lane 5, PDGF + desoxycorticosterone (1×10^{-8} M) and lane 6, PDGF + pregnenolone (1×10^{-8} M). (b) Representative Northern-blot of the dose-dependent inhibition of the PDGF-AA- or -BB-induced transcription of the VEGF mRNA by hydrocortisone (prednisolone); lane 1, stimulus alone (PDGF-AA: 10 ng/ml, PDGF-BB: 2.5 ng/ml, respectively); lane 2–4, stimulus plus prednisolone (lane 2: 1×10^{-10} M, lane 3: 1×10^{-8} M, lane 4: 1×10^{-6} M). Control = cells stimulated with vehicle alone.

cultivated human vascular smooth muscle cells. These results are in agreement with the recently reported upregulation of VEGF mRNA by PDGF-BB in vascular smooth muscle cells (Stavri et al., 1995). In contrast, the transcription of HLA-β gene was not affected by any of the PDGF-isoforms (Fig. 1a). Following kinetic studies, the onset of transcription was observed 60 min after stimulation with PDGF, attained a peak between 2 and 4 h and declined thereafter to basal levels (Fig. 1b). The addition of 1×10^{-6} M actinomycin D abolished the transcription of VEGF, indicating that the observed mRNA signal is due to de novo transcription of mRNA encoding VEGF and not due to accumulation of the respective mRNA (Fig. 1c).

The mitogen-induced transcription of VEGF mRNA was subsequently followed by de novo synthesis and secretion of the protein by vascular smooth muscle cells (Fig. 2). The above results were confirmed in duplicate experiments in six primary human vascular smooth muscle cells lines.

3.2. Corticosteroids inhibit the PDGF-induced VEGF mRNA expression and VEGF protein secretion by human vascular smooth muscle cells

In the presence of cortisone, hydrocortisone or dexamethasone (1×10^{-8} to 1×10^{-12} M) the mitogen-dependent (all isoforms) transcription of VEGF was completely abolished, whereas the transcription of control gene (HLA-β) was unaffected (Fig. 3a). There were no statistically significant differences observed on the above inhibitory effect of the glucocorticoids when compared at equimolar concentrations. The inhibitory effect of the corticosteroids tested was dose-dependent, between 1×10^{-8} to 1×10^{-12} M (Fig. 3b). In contrast, the two cholesterol precursors of the corticosteroids: desoxycorticosterone or pregnenolone (1×10^{-8} to 1×10^{-12} M) did not produce any statistically significant effect on the PDGF-induced VEGF mRNA expression (Fig. 3a).

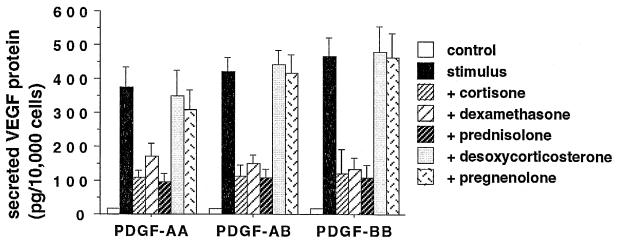


Fig. 4. Effect of corticosteroids and their cholesterol precursors on PDGF-dependent secretion of VEGF in cultivated human vascular smooth muscle cells. Secretion of VEGF protein was determined by EIA 12 h after the addition of PDGF-AA (10 ng/ml), PDGF-AB (5 ng/ml) or PDGF-BB (2.5 ng/ml) in the presence and absence of either cortisone, hydrocortisone, or dexamethasone (all at a concentration of 1×10^{-9} M), or their cholesterol precursors, desoxycorticosterone or pregnenolone (1×10^{-8} M). Each bar represents the mean ± S.E. of six independent experiments.

Similar to their inhibitory effect on transcription, the glucocorticoids diminished the de novo synthesis and release of VEGF protein stimulated by any of the three PDGF isoforms (Fig. 4). There were no statistically significant differences on the potencies of the glucocorticoids on the de novo synthesis of VEGF. The inhibitory effect of steroids tested was dose-dependent (1×10^{-8} to 1×10^{-12} M), as is typically shown in Fig. 4 on the PDGF-BB-induced release of VEGF protein. In contrast to the above described inhibitory effect of cortisone, hydrocortisone or dexamethasone, the cholesterol precursors of the corticosteroids: desoxycorticosterone or pregnenolone (1×10^{-8} to 1×10^{-12} M) did not affect VEGF protein secretion by human vascular smooth muscle cells (Fig. 4).

4. Discussion

4.1. The importance of inhibitors of angiogenesis

Angiogenesis inhibitors have potential therapeutic applications in disease states associated with angiogenesis, such as diabetic retinopathy, retrolental fibroplasia, psoriasis, scleroderma, gingivitis and tumorigenesis (reviewed by Maragoudakis et al., 1992; Auerbach and Auerbach, 1994; Folkman, 1995). Numerous angiostatic compounds have been identified (reviewed by Auerbach and Auerbach, 1994) some of which are in phase I and II clinical trials, such as the fumagillin-derivative AGM1470, interferon-γ-2a and carboxy-amino-triazole (reviewed by Folkman, 1995). The inhibitors of angiogenesis act by vastly diverse mechanisms (reviewed by Auerbach and Auerbach, 1994). They may act, for example, by inhibiting basement membrane biosynthesis, as are the agents 8,9-dihydroxy-7-methylbenzo(β)-quinolizinium bromide and tricyclodecan-9-ylxanthate (Maragoudakis et al., 1990, 1993) or by inhibiting platelet adhesion, as are the nitric oxide-releasing vasodilators isosorbide-5-mononitrate and dinitrate (Pipili-Synetos et al., 1995), or by an as yet unknown mechanism of action, as the extensively studied combination of heparin with cortisone or hydrocortisone (Folkman et al., 1983; Folkman and Ingber, 1987; Maragoudakis et al., 1989).

4.2. VEGF as a key target for inhibiting angiogenesis

The available evidence of the last 5 years indicates that VEFG may be a major regulator for inducing neovascularization and a key target for inhibiting angiogenesis. Inhibitors of VEGF described so far include: (a) high-affinity RNA ligands which inhibit VEGF binding to its receptors (Jellinek et al., 1994), (b) Rp-cAMP, a cAMP antagonist, which suppressed upregulation of VEGF mRNA by prostaglandin E_2 in osteoblasts and in bone tissue (Harada et al., 1994), (c) antibodies directed against VEGF, which can

inhibit the growth of a variety of VEGF producing tumors (Neufeld et al., 1994) and prevent the chemotaxis of human umbilical vein endothelial cells and the mitogenic activity of bovine adrenal capillary endothelial cells induced by rheumatoid arthritis synovial fluid (Koch et al., 1994) and (d) a retrovirus encoding a dominant-negative mutant of the Flk-1/VEGF receptor which was used to infect endothelial target cells and prevent tumor growth in nude mice (Millauer et al., 1994). Here we report that corticosteroids may be another class of VEGF inhibitors.

4.3. Corticosteroids but not their precursors inhibit the PDGF-induced transcription of the VEGF gene and secretion of VEGF protein

In this study we present evidence that PDGF (all three isoforms) induced the transcription and secretion of VEGF in vascular smooth muscle cells obtained from the human pulmonary aorta. These results are in agreement with the recently reported upregulation of VEGF by PDGF-BB in vascular smooth muscle cells (Stavri et al., 1995). Here we show that cortisone, hydrocortisone and dexamethasone (1×10^{-8} to 1×10^{-12} M) can inhibit in a dose-dependent manner the PDGF-induced transcription of the VEGF gene as well as the PDGF-dependent secretion of VEGF protein.

In contrast, the cholesterol precursors of the corticosteroids: desoxycorticosterone and pregnenolone did not produce any statistically significant effect at the above concentration range. Our results are in line with: (a) the reports that dexamethasone can suppress the upregulation of VEGF mRNA by prostaglandin E_2 in osteoblasts and in bone tissue (Harada et al., 1994), (b) the reported efficacy of high-dose dexamethasone in the treatment of neoplastic brain edema (Criscuolo, 1993) and (c) the inhibitory effect of hydrocortisone on microvascular endothelial cell migration (Stokes et al., 1990).

Our findings indicate that the anti-angiogenic action of cortisone, hydrocortisone and dexamethasone, when used alone or in combination with heparin, may be attributed, at least in part, to the ability of these corticosteroids to abolish the PDGF-induced transcription of VEGF mRNA and secretion of VEGF protein by vascular smooth muscle cells. In this context, it is of interest to report that this action of corticosteroids is not confined to vascular smooth muscle cells. In preliminary experiments we observed a similar effect of these corticosteroids on PDGF-induced transcription of VEGF mRNA and secretion of VEGF protein by primary fibroblast cell lines from human skin and pulmonary arteries. This finding is currently being substantiated by studies which are further extended to clarify if the inhibitory effect of the corticosteroids we described above is specific to VEGF or if it affects other growth factors known to be involved in angiogenesis, such as basic fibroblast growth factor.

4.4. Corticosteroids reduce edema by inhibiting the PDGF-induced transcription of VEGF mRNA and secretion of VEGF protein by vascular smooth muscle cells

One of the important physiological and pharmacological effects of corticosteroids is their capacity to prevent or suppress the development of the manifestations of inflammation, including edema and capillary dilatation, by inhibiting the release from different cell sources of mediators of inflammation, such as arachidonic acid and its metabolites (prostaglandins and leukotrienes), platelet activating factor, tumor necrosis factor, interleukin-1 and plasminogen activator (Haynes, 1992). Here, we have presented evidence that, in addition to the above, the anti-inflammatory action of corticosteroids may also be attributed to their ability to inhibit the PDGF-induced transcription of VEGF mRNA and secretion of VEGF protein, which enhances vascular permeability (Keck et al., 1989; Leung et al., 1989) and edemagenesis (Criscuolo, 1993). This alternative anti-inflammatory action of cortisone, hydrocortisone and dexamethasone is strengthened by the observation that the cholesterol precursors of the corticosteroids desoxycorticosterone and pregnenolone, which have no anti-inflammatory activity (Haynes, 1992), are devoid of the above inhibitory action on VEGF expression.

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