Vascular endothelial growth factor in the rat pituitary: differential distribution and regulation by estrogen

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Abstract

Vascular endothelial growth factor (VEGF), an endothelial cell mitogen and permeability factor, participates in tumor angiogenesis, but less is known about its regulation or function in normal vascular homeostasis. In the uterus, which undergoes cyclic changes in its vasculature, VEGF is induced by estrogen. Since the pituitary gland contains highly permeable capillaries and is estrogen-responsive, our objectives were to localize VEGF expression within the pituitary and to determine whether it is regulated by estrogen in both the pituitary and the somatotrope cell line, GH₃.

Ovariectomized rats were injected with estradiol, and pituitaries and uteri were subjected to *in situ* hybridization or quantitative reverse transcription-polymerase chain reaction (RT-PCR). VEGF expression was strong and punctate in the neural lobe, weaker and diffuse in the anterior lobe and undetectable in the intermediate lobe. Two VEGF isoforms, 164 and 120, were detected in all

tissues. In the posterior pituitary, VEGF expression was 3- to 6-fold higher than in the anterior pituitary or uterus and was unaltered by estrogen. In contrast, anterior pituitary VEGF was induced by estrogen within 1 h, peaked at 3 h, and returned to basal levels by 24 h. Similar dynamics, albeit 10-fold higher, were seen in the uterus. Translated VEGF proteins were detected by Western blot in both the anterior pituitary and uterus. GH₃ cells also showed a dose- and time-dependent induction of VEGF expression by estrogen.

In conclusion: (1) VEGF expression is higher in the neural lobe than in the anterior lobe and is undetectable in the intermediate lobe, (2) the expression of VEGF164 and VEGF120 is rapidly upregulated by estrogen in the anterior pituitary but is unchanged in the posterior pituitary, and (3) the pituitary lactotrope cell line, GH₃, also increases VEGF expression in response to estradiol.

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Introduction

Angiogenesis is a complex process involving remodeling of the extracellular matrix and endothelial cell proliferation that results in the formation of new blood vessels. Active angiogenesis occurs during embryonic development as well as during wound healing and neoplasia (Breier *et al.* 1992, Folkman & Shing 1992). In normal adult tissues, endothelial cells are quiescent, with the exception of the uterus and ovary, which undergo neovascularization and altered vascular permeability during the reproductive cycle (Reynolds *et al.* 1992).

Vascular endothelial growth factor (VEGF), a 43–46 kDa heparin-binding dimeric glycoprotein, is a potent mitogen with high specificity for the endothelia. The VEGF gene undergoes alternative splicing to yield several isoforms (Houck *et al.* 1992, Ferrara & Davis-Smyth 1997). In rodents, monomers of 188, 164, and 120 amino

acids have been identified (Fig. 1). VEGF exerts its effects by binding to two high-affinity receptor tyrosine kinases, flt-1 and flk-1/KDR, which are primarily found on endothelial cells (De Vries *et al.* 1992, Quinn *et al.* 1993). A recently discovered third receptor, neuropilin-1, selectively binds to VEGF164 and is not confined to endothelial cells (Soker *et al.* 1998). In addition to its mitogenic function, VEGF is the most powerful of the known mediators of vascular permeability (Senger *et al.* 1990, Connolly 1991). VEGF increases vascular permeability by inducing and maintaining the fenestrae, which are pores in the endothelial cell membrane that enable exchange of water and solutes (Roberts & Palade 1995, 1997, Esser *et al.* 1998).

The pituitary gland contains a dense network of capillaries that are characterized by a single layer of thin, highly fenestrated endothelial cells (Farquhar 1961, Gross *et al.* 1987). This permeable endothelium allows for the

exchange of hormones between the blood and the pituitary. Unlike the anterior and neural lobes, the intermediate lobe contains few, if any, capillaries (Adams *et al.* 1963, Porter *et al.* 1973). The factors that are responsible for the maintenance and regulation of the pituitary vasculature have not been well defined. Although VEGF was originally isolated from folliculo-stellate cells of the bovine pituitary (Gospodarowicz *et al.* 1989), there is only limited information on its distribution, regulation and function within the pituitary gland. In the ovine pituitary, VEGF expression was detected primarily in the pars tuberalis, and flt-1 was localized to the capillary endothelia (Jabbour *et al.* 1997). In the rat pituitary, the VEGF protein was found in untreated females and its level increased during the development of estrogen-induced prolactin-secreting tumors (Banerjee *et al.* 1997). These authors also reported that both microvessel density and flk-1/KDR expression increased in the hyperplastic pituitaries.

A rapid and transient induction of VEGF by estrogen has been well documented in the uterus (Charnock-Jones *et al.* 1993, Cullinan-Bove & Koos 1993, Hyder *et al.* 1996, Reynolds *et al.* 1998). Although the pituitary gland is also an established target of estrogens, potential short-term effects of estrogen on VEGF gene expression have not been investigated. The overall goal of this study was to localize VEGF expression throughout the rat pituitary and to determine its regulation by estrogen. As an experimental model, we have selected the estrogen-sensitive Fischer 344 (F344) rat for the *in vivo* studies and the estrogen-responsive rat somatolactotrope cell line GH₃ for the *in vitro* studies (Steinmetz *et al.* 1997). The objectives were: (1) to compare the expression of the various VEGF isoforms at both the RNA and protein levels in the pituitary gland and uterus, (2) to analyze the dynamics of estrogen-induced VEGF expression in the pituitary and uterus, and (3) to determine whether GH₃ cells express VEGF which is regulated by estrogen.

Materials and Methods

Animals

All studies were conducted with an institutionally approved protocol in accordance with the USPHS Guide for the Care and Use of Laboratory Animals. Adult (8–10 weeks old) Fischer 344 female rats (Harlan Laboratories, Indianapolis, IN, USA) were ovariectomized 14 days prior to experiments. Rats were maintained on a 12-h light–darkness schedule and food and water were available *ad libitum*. For hormone treatment, rats were injected s.c. with 17 β -estradiol (10 μ g/kg) or sesame oil vehicle and killed after 1, 3, 6, or 24 h. For *in situ* hybridization, whole pituitaries and uteri were collected. For reverse transcription-polymerase chain reaction (RT-PCR) analysis, uteri were removed and anterior (AP) and posterior (PP) pituitaries were separated.

Figure 1 Schematic representation of the VEGF mRNA and its isoforms, VEGF188, VEGF164, and VEGF120, that arise from alternative splicing. Arrows indicate the locations of the RT-PCR primers capable of detecting all three VEGF isoforms.

Prolactin determination by RIA

Prolactin (PRL) levels in serum from control and estrogen-treated rats were determined by RIA using an NIDDK rat PRL kit with RP-3 as a reference preparation, as described previously (Steinmetz *et al.* 1997).

In situ hybridization

In situ hybridization was performed as previously described (Allen *et al.* 1997). Briefly, frozen sections of pituitary and uteri (10 μ m) from 2–3 rats per treatment were mounted on silane-coated slides and fixed in 4% paraformaldehyde. Slides were dehydrated with ethanol and stored at -80°C . Prior to hybridization, slides were rehydrated, treated with proteinase K (20 μ g/ml) and post-fixed in 4% paraformaldehyde. Slides were then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine and dehydrated.

The VEGF riboprobe was synthesized from a VEGF164 cDNA that was RT-PCR amplified from rat pituitary RNA and cloned into the EcoRI and HindIII sites of Bluescript SK⁺ vector (Stratagene, La Jolla, CA, USA). After cloning, the VEGF164 insert was confirmed by sequencing. Both sense and antisense riboprobes were labeled with ³⁵S using a Promega (Madison, WI, USA) *in vitro* transcription kit and were purified on Sephadex G50 spin columns. Slides were incubated with either antisense or sense riboprobes (750 000 c.p.m./section) in hybridization buffer at 55 $^{\circ}\text{C}$ for 14–17 h. The sections were subjected to a high stringency wash consisting of 50% formamide, 2 $\times$ SSC and 10 mM dithiothreitol at 65 $^{\circ}\text{C}$ for 30 min, followed by several washes. To digest nonspecifically bound probe, sections were treated with 20 μ g/ml RNase A and 33 U/ml RNase T₁ for 30 min. The slides were dipped in Kodak NTB2 photographic emulsion (Eastman Kodak, Rochester, NY, USA) and developed after 5–7 days.

RT-PCR

Total RNA was extracted from individual uteri and AP and from 2–3 pooled PP using Tri-Reagent (Molecular

Research Center, Cincinnati, OH, USA). The RNA (5 µg) was reverse transcribed using Superscript II reverse transcriptase and random hexamers (Gibco BRL, Gaithersburg, MD, USA). All PCR reactions were carried out using the following primers for VEGF: antisense primer 5'-GCTCTCTTGGGTGCACTGGA-3', and sense primer 5'-CACCGCCTTGGCTTGTACA-3' (Nakamura *et al.* 1996) with expected sizes of 635, 563, and 431 bp for amplification of VEGF188, VEGF164, and VEGF120 respectively (see Fig. 1 for location of the VEGF primers). Primers for the internal control, ribosomal protein L19 (RPL19), were included in each reaction: antisense primer 5'-CGAAATCGCCAATGCCAACTC-3', and sense primer 5'-TGCTCCATGAGAATCCGCTTG-3' with an expected size of 333 bp.

Optimal PCR conditions for the VEGF gene were first established by varying the RNA concentration and the number of cycles. Thereafter, 200 ng of the RT mixture were used under the following PCR conditions: denaturation at 95 °C for 30 s, annealing at 65 °C for 45 s, and extension at 72 °C for 45 s, for 28 cycles. PCR products were separated on a 1% agarose gel, visualized using ethidium bromide, photographed and analyzed by scanning densitometry (Scion Image Software, Frederick, MD, USA).

Western blot analysis

Individual anterior pituitaries or uteri from control or estrogen-treated rats (10 µg/kg for 6 h) were homogenized in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) containing the following protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride (Sigma, St Louis, MO, USA), 20 mM sodium molybdate (Sigma), and 50 µg/ml lima bean trypsin inhibitor (Worthington Biochemical, Freehold, NJ, USA). Samples were centrifuged at 13 000 × *g* for 10 min at 4 °C and protein content was determined by the Pierce BCA kit (Rockford, IL, USA). For each tissue, 50 µg proteins were resolved by SDS-polyacrylamide gel electrophoresis (12% gel). Recombinant human VEGF164 and mouse VEGF120 (30 ng; R & D Systems, Minneapolis, MN, USA) were used as positive controls. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA), and after blocking in 5% nonfat milk for 1 h, the membranes were incubated with 1 µg/ml polyclonal anti-human VEGF antisera (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 h. The membranes were then incubated with a peroxidase-conjugated horse anti-goat IgG (1:10 000; Vector Labs, Burlingame, CA, USA) for 1 h. Protein bands were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). The specificity of the antibodies was confirmed by preabsorption with the synthetic peptide (Santa Cruz Biotechnology).

GH₃ cell culture

GH₃ cells were maintained in Ham's F-10 medium supplemented with 15% heat-inactivated gelding serum (Central Biomedica, Lenexa, KS, USA) that has undetectable estrogen levels. Cells (50 000/well) were plated in 24-well plates coated with protamine (Sigma) and Nu-Serum (Collaborative Research, Bedford, MA, USA) as previously described (Steinmetz *et al.* 1997). The cells were incubated for 2–3 days in serum-free media composed of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-10 (50/50) supplemented with ITS+premix (Collaborative Research) and penicillin/streptomycin. Under these conditions, there is minimal cell proliferation. For estrogen treatment, cells were incubated with 0.1, 1 or 10 nM estradiol for 24 h or with 1 nM estradiol for 1, 3, 6 or 24 h. Control cells were incubated with 0.001% ethanol vehicle. At the end of the experiment, cells pooled from 4 wells were lysed with Tri-Reagent and RT-PCR performed as described above.

Data analysis

For comparison of the two VEGF isoforms in the different tissues by RT-PCR, data were calculated as a density ratio of VEGF/RPL19. For the time-course and GH₃ experiments, data were presented as percentage of vehicle control, with the control being set at 100%. Each treatment included 5–12 rats and the results were expressed as the mean ± S.E.M. The GH₃ cell incubation studies included 5–7 independent experiments. Statistical analysis was carried out by a one-way ANOVA followed by a Dunnett's *post hoc* test.

Results

Distribution of VEGF mRNA within the pituitary gland and uterus

In situ hybridization was used to localize VEGF mRNA in the pituitary gland and uterus from either control or estrogen-treated rats. A 3-h estradiol treatment was chosen because this time-point showed maximal VEGF induction in preliminary experiments. A pituitary immunostained for proopiomelanocortin (POMC), a marker for anterior lobe corticotropes and intermediate lobe melanotopes, is included in Fig. 2, left panel, to illustrate the definition between the three lobes. Fig. 2, middle panels, show a much higher expression of VEGF in the neural lobe than in the anterior lobe. The VEGF signal was strong and punctate in the neural lobe, whereas the expression pattern for VEGF in the anterior lobe was more diffuse. Interestingly, VEGF mRNA was undetectable in the intermediate lobe. The specificity of the VEGF antisense riboprobe was demonstrated by the lack of signal in a

Figure 2 Localization of VEGF expression within the pituitary gland shown by *in situ* hybridization. Pituitary cryosections were hybridized with ^{35}S -labeled VEGF antisense or sense riboprobes. The left panel shows a pituitary immunostained for POMC to highlight the distinction between the three lobes. The middle panels show control and estrogen-treated ($10\text{ }\mu\text{g/kg}$ for 3 h) pituitaries hybridized with the antisense probe. The right panel shows a control pituitary hybridized with the sense probe. Magnification: $\times 4$. IL: intermediate lobe; NL: neural lobe; AL: anterior lobe. This is a representative experiment that was repeated 2–3 times.

Figure 3 Localization of VEGF expression within the uterus. Cryosections of control (left panel) and estrogen-treated (right panel) uteri were hybridized with a ^{35}S -labeled antisense VEGF probe. See Fig. 2 for other details. Magnification: $\times 20$.

pituitary hybridized with the sense riboprobe (Fig. 2, right panel).

In situ hybridization was next performed on the uterus (Fig. 3). VEGF mRNA levels were extremely low in the control uterus, but were markedly increased upon estrogen stimulation. The VEGF transcripts were highly expressed in the glandular and luminal epithelium, with some expression seen in the myometrium and stroma. The sense

riboprobe showed no hybridization signal throughout the uterus (data not shown).

Optimization of PCR conditions

Optimal RT-PCR conditions for VEGF were established by varying the number of cycles and amount of RNA. Although the PCR primers are capable of detecting all

Figure 4 Optimization of the conditions for RT-PCR analysis of VEGF mRNA in untreated anterior pituitaries. The upper panels are representative RT-PCR gels of increasing cycle number (left) and RNA amount (right). Both gels show the detection of VEGF164 (563 bp), VEGF120 (431 bp), and RPL19 (333 bp), an internal control included in each reaction. Lanes 1–5 (left gel) designate 24, 26, 28, 30, and 32 cycles respectively. Lanes 1–5 (right gel) designate 30–500 ng RNA, respectively, as shown in the graph below. L: 100 bp ladder. The lower panels show the densitometric values. The left panel depicts an increase in VEGF164, VEGF120 and RPL19 between cycles 26 and 30. The right panel shows a dose-dependent increase in all three products.

VEGF isoforms, only two isoforms, VEGF164 (563 bp) and VEGF120 (431 bp), were apparent in the anterior pituitary (Fig. 4, upper panels). The optical density of the two isoforms and the internal control, RPL19, increased in an exponential manner (Fig. 4, lower left panel). Twenty-eight cycles were chosen for subsequent experiments. Figure 4, lower right panel, shows a linear increase in optical density between 125 and 500 ng RNA for the three genes. Because pituitaries from control rats were used for the optimization, an RNA amount that would allow determination of both control and estrogen-induced increases was selected. Therefore, 200 ng RNA and 28 cycles were used for the next set of experiments. The identity of the RT-PCR products was confirmed by both restriction enzyme analysis and sequencing (data not shown).

Comparison of the VEGF isoforms in the anterior pituitary, posterior pituitary, and uterus

The relative levels of VEGF164 and VEGF120 in the anterior pituitary, posterior pituitary (neurointermediate

lobe) and uterus were compared using RT-PCR. Tissues from rats treated with estrogen or vehicle for 3 h were used. As in the pituitary, only two VEGF isoforms, 164 and 120, were detected in the uterus. As evident in Fig. 5, the expression of VEGF164, and to a lesser extent VEGF120, is significantly higher ($P < 0.05$) in the posterior pituitary than in either the anterior pituitary or uterus under unstimulated conditions. The expression of either VEGF isoform in the posterior pituitary was not altered by estrogen. In contrast, VEGF expression was highly up-regulated by estrogen in the uterus and to a lesser extent in the anterior pituitary ($P < 0.05$). Serum PRL levels in rats treated with estrogen for 3 h (64.3 ± 9.6 ng/ml) were significantly higher ($P < 0.05$) than those in vehicle-treated rats (17.7 ± 2.1 ng/ml).

Rapid and transient induction of VEGF expression in the pituitary and uterus by estrogen

The time course of the VEGF induction by estrogen in the anterior pituitary and uterus was determined by RT-PCR (Fig. 6). Within 1 h after a single injection of estradiol,

Figure 5 Comparison of the relative expression of VEGF164 (upper panel) and VEGF120 (lower panel) in the anterior pituitary (AP), posterior pituitary (PP), and uterus (UT). Both control and tissues treated with estrogen for 3 h were analyzed by RT-PCR and presented as the ratio of VEGF/RPL19. Each value is the mean $\pm$ S.E.M. of 5–12 rats. See Figs 2 and 4 for other details.

there was a rapid induction of the two VEGF isoforms in both tissues ($P < 0.05$). Maximal induction was observed at 3 h ($P < 0.05$), followed by a decline at 6 h ($P < 0.05$), and a return to basal levels by 24 h. The estradiol-induced increase in VEGF mRNA expression was 8- to 10-fold lower in the anterior pituitary than in the uterus.

Translated VEGF proteins in the anterior pituitary and uterus

To determine whether the VEGF isoforms are translated, Western blot analysis was used. Figure 7 shows the presence of VEGF164 and VEGF120 as 21–23 kDa and 14–15 kDa proteins, respectively, in both tissues. VEGF164 was seen in the pituitary and uterus under both control and estrogen-treated conditions (6 h). On the other hand, VEGF120 was detected in the pituitary under

both conditions, but was evident only in the estrogen-treated uterus. Glycosylated forms of VEGF may account for the appearance of additional bands. Due to the minute size of the rat posterior pituitary, and inconsistent results, this tissue was not included in this part of the study. No immunoreactive bands were observed when the antibody was preabsorbed with the synthetic peptide (data not shown). Coomassie Blue staining of the gel demonstrated equal sample loading (data not shown).

Induction of VEGF expression by estradiol in GH₃ cells

Figure 8 demonstrates that estradiol increased VEGF expression in GH₃ cells in a time- and dose-dependent fashion. The left panel shows that 1 nM estradiol increased VEGF164 expression 3-fold above control ($P < 0.05$) and there was no further rise with the 10 nM dose. On the other hand, VEGF120 increased in a linear manner in response to all three doses of estradiol ($P < 0.05$). When the cells were incubated with 1 nM estradiol for various times, VEGF164 increased significantly ($P < 0.05$) after 3 h, declined at 6 h ($P < 0.05$) and remained elevated above control at 24 h ($P < 0.05$). The estrogen-induced rise in VEGF120 showed an attenuated time-dependent expression pattern.

Discussion

We are reporting a differential distribution of VEGF mRNA within the rat pituitary gland under basal conditions. The expression of VEGF is strong and punctate in the neural lobe, weaker and more diffuse in the anterior lobe and undetectable in the intermediate lobe. Two isoforms of VEGF, 164 and 120, are present at both the RNA and protein levels in the pituitary gland and uterus. Estrogen upregulates VEGF expression in the anterior lobe in a rapid and transient manner but does not affect VEGF in the neural lobe. The dynamics of the estrogen-induced rise in VEGF mRNA in the anterior pituitary resemble that in the uterus but the levels are significantly lower in magnitude. Involvement of lactotropes is suggested by the induction of VEGF expression in GH₃ cells by estrogen, that is similar in time course and magnitude to that observed in the anterior pituitary.

The unexpected finding of high basal expression of VEGF within the neural lobe, as seen by *in situ* hybridization (Fig. 2), raises the question as to the cellular origin of VEGF. The neural lobe contains numerous axon terminals, modified astroglial cells named pituicytes, and endothelial cells. VEGF has been detected in retinal astroglial cells during neonatal development (Stone *et al.* 1995), and in gliomas (Plate *et al.* 1992), but not in adult glia under normal conditions. Unstimulated endothelial cells express little, if any, VEGF, but can greatly increase

Figure 6 Time course of the increase in VEGF expression in the anterior pituitary (left panels) and uterus (right panels) after a single injection of estrogen (10 µg/kg). The upper panels show representative RT-PCR gels from control (C) and estrogen-treated (E2) anterior pituitaries (10 µg/kg for 3 h; left panel) and uteri (right panel). In the lower panels the VEGF mRNA detected by RT-PCR was normalized to RPL19 and presented as a percentage of control values, with the control being 100%. Each value is the mean $\pm$ S.E.M. of 6–8 rats.

its production under abnormal situations such as tumor formation and vascular disease (Folkman 1995). Should either pituicytes or endothelia be identified as the source of the constitutive expression of VEGF in the neural lobe, this would represent an atypical situation. Unlike the anterior pituitary, there was no rapid induction of VEGF expression by estradiol in the posterior pituitary. Similarly, we previously reported that estradiol induced *c-fos* expression in the anterior, but not the neural, lobe (Allen *et al.* 1997).

Clearly, the intermediate lobe is devoid of VEGF expression (Fig. 2). This lobe contains very few capillaries, with the short portal vessels traversing this lobe to connect the anterior and neural lobes. The avascularity of the intermediate lobe is rather unusual and the mechanism responsible for such limited vascularization is unknown. The primary cell type within the intermediate lobe is the melanotrope, which is under negative regulation by dopamine (Ben-Jonathan 1985). Evidence suggests that removal of dopaminergic inhibition by pituitary stalk

section (Adams *et al.* 1963), dopaminergic antagonists (Chronwall *et al.* 1987), or pituitary grafting (Gon *et al.* 1987), results in marked hyperplasia and hypertrophy of the intermediate lobe. Whether such enlargement is also associated with neovascularization has not been investigated. The possibility that the low capillary density within the intermediate lobe is due to dopaminergic inhibition of VEGF expression by the melanotropes is currently under investigation.

The uterus was included in these studies to validate both the *in situ* hybridization and RT-PCR techniques. The unstimulated uterus showed little hybridization signal with the antisense VEGF probe (Fig. 3). However, following estrogen administration, a very strong expression was evident, especially in the glandular and luminal epithelium. A dramatic induction of VEGF expression in the uterus by estradiol was previously reported by Hyder *et al.* (1996), except that VEGF expression was stronger in the stroma. The difference in the regional distribution of VEGF between the two studies is probably due to the use

Figure 7 VEGF proteins in control (C) or estrogen-treated (E2) anterior pituitaries (A. Pit) and uterus as determined by Western blot analysis. Ovariectomized F344 rats were treated with estradiol (10 µg/kg) or vehicle (control) for 6 h. Proteins (50 µg) were resolved on SDS-PAGE (12% gel), transferred to a nitrocellulose membrane, and incubated with anti-VEGF antisera (1 µg/ml). Enhanced chemiluminescence was used to visualize the proteins. Recombinant VEGF proteins (30 ng) were used as positive controls. Arrows on the left indicate location of the protein markers. This is a representative experiment which was repeated 2–3 times.

of prepubertal rats in their study and the use of adult rats in the present study.

Because *in situ* hybridization cannot discriminate between the VEGF isoforms, an RT-PCR analysis was utilized. Although the primers were capable of detecting all the splice variants, only two isoforms, VEGF164 and VEGF120, were found throughout the pituitary gland or uterus (Fig. 5). VEGF164, and to a lesser extent VEGF120, were highly expressed in the posterior pituitary, which is composed of both neural and intermediate lobes. Since VEGF is undetected in the intermediate lobe, its expression in the posterior pituitary is attributed to the neural lobe only. The function served by either isoform within the pituitary is unknown. Although both VEGF164 and VEGF120 can induce endothelial cell proliferation and increase permeability, they differ in their heparin-binding

properties, potency, and tissue distribution (Ferrara & Davis-Smyth 1997). For example, the higher molecular weight isoforms appear to be more mitogenic, bind to heparin more tightly and can be found in the extracellular matrix, whereas VEGF120 is a freely soluble protein (Park *et al.* 1993, Ferrara & Davis-Smyth 1997). The dramatic induction of VEGF by estrogen in the uterus has previously been reported by other investigators and was included in this study as a comparison with the pituitary.

VEGF expression was detected throughout the anterior pituitary and was upregulated by estrogen in a rapid and transient manner (Fig. 6). Although several cell types within the anterior pituitary may express VEGF, the identity of the cells that increase VEGF expression in response to estrogen remains unknown. We speculate that lactotrope as well as folliculo-stellate cells, which together comprise 50–60% of all pituitary cell types (Chen 1987, Allen *et al.* 1997), might be involved. The lactotrope is implicated, based on the reports that the estrogen-induced prolactin secreting tumors in the F344 rat are associated with upregulation of VEGF (Banerjee *et al.* 1997) and development of a new arterial blood supply (Elias & Weiner 1984, Schechter *et al.* 1987). This is supported by our findings that VEGF is expressed in two lactotrope cell lines, GH₃ and MMQ cells (Ochoa A L, Ben-Jonathan N, unpublished observation), and is increased by estrogen. In fact, the rapid induction of the two VEGF isoforms by estrogen in GH₃ cells (Fig. 8) is similar to that seen in the anterior pituitary (Fig. 6), and cannot be attributed to an induction of cell proliferation by estrogen. As reported by us previously (Steinmetz *et al.* 1997), incubation of GH₃ cells with estrogen for 24 h resulted in only a 20–25% increase in cell number.

The folliculo-stellate cell, a non-hormone producing cell, is known to express VEGF (Gospodarowicz *et al.* 1989). It expresses both estrogen receptors (Mitchner *et al.*

Figure 8 Induction of VEGF expression in GH₃ cells by estrogen. The left panel shows dose-dependent increase in VEGF expression after treatment with 0.1–10 nM estradiol for 24 h. The right panel shows the time course of VEGF induction by 1 nM estradiol for 1–24 h. See Fig. 7 for other details. Each value is the mean ± S.E.M. of 5–7 independent experiments.

1998) and responds to estrogen by increasing *c-fos* expression (Allen *et al.* 1997) and undergoing morphological changes (Schechter *et al.* 1988). Combined immunocytochemistry/*in situ* hybridization, presently being undertaken in our laboratory, will enable us to identify which pituitary cell types increase VEGF expression in response to estrogen.

The rapid induction of VEGF in the pituitary, GH₃ cells and uterus suggests that the VEGF promoter is directly activated by the estrogen receptor. Although there are no consensus estrogen response elements (ERE) within the VEGF promoter, there are two ERE binding sites in the 5' and 3' untranslated regions (Hyder *et al.* 1999). Further analysis of the VEGF gene is necessary to determine the mechanism of the estrogenic action. In addition to a direct effect, we cannot discount the possibility of indirect effects of estrogen on other genes within the VEGF-producing or neighboring cells. An indirect action via hypothalamic factors is also conceivable.

It is important to consider the potential functions of VEGF within the pituitary gland. Unlike the uterus, where estrogen markedly increases blood flow and tissue permeability, it is not known whether such changes also occur in the pituitary gland. A plausible function for VEGF within the normal pituitary is as a permeability factor. Both the anterior and neural lobes contain a dense capillary network that is characterized by the presence of fenestrated endothelium, similar to that found in other endocrine organs (Farquhar 1961, Gross *et al.* 1987). Fenestrae are circular pores of approximately ~60 nm in diameter which traverse the endothelial cells (Palade 1988), allowing an exchange of solutes between the endothelium and surrounding tissue. VEGF was reported to induce fenestrations in a continuous, non-permeable, endothelium (Roberts & Palade 1995, 1997, Esser *et al.* 1998). The persistent expression of VEGF in the pituitary, especially in the neural lobe, may be responsible for the maintenance of the highly permeable endothelium. In fact, the density, morphology and blood flows of the capillaries within the two lobes differ, with the neural lobe containing twice as many capillaries with smaller diameter than the anterior lobe (Gross *et al.* 1993).

Our studies revealed a differential distribution of VEGF within the pituitary and a rapid upregulation by estrogen. However, little is known about the effects of short-term estrogen treatment on the pituitary vasculature and the function(s) of VEGF in the pituitary gland. Future studies should characterize angiogenic and angiostatic factors in the pituitary and their effects on the morphology and function of the microvessels.

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