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Vascular Endothelial Growth Factor Is Up-Regulated *in Vitro* and *in Vivo* by Androgens

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Evidence from pathophysiological studies support the concept that embryonic development, tumor progression, and hormonally-regulated tissue masses such as adult prostate and corpus luteum are angiogenesis-dependent. We examined if the prostatic expression of vascular endothelial growth factor (VEGF), the major regulator of normal and pathological angiogenesis, was regulated by testosterone. Northern blot of VEGF messenger ribonucleic acid (mRNA) extracted from a human immortalized epithelial prostatic cell line (PNT1) showed that dihydrotestosterone (DHT) up-regulated VEGF mRNA at a level comparable to that observed upon exposure to growth factors. Polymerase chain reaction of reverse transcribed mRNA demonstrated that the ratio of the two splice variants encoding the 121 and 165 isoforms of VEGF were not affected by DHT. VEGF biological activity, measured in the conditioned medium by radio receptor assay, was increased by DHT. Injection of testosterone in adult rats induced a transient increase of the ventral lobe weight and the specific activity of prostatic VEGF, leading to a 7-fold increase in the prostate content of VEGF. © 1998 Academic Press

Key Words: **androgens; vascular endothelial growth factor; angiogenesis; prostate.**

The progressive growth of a malignant tumor requires an adequate blood supply, which is provided by newly formed vessels. Abundant evidence supports the concept that prostate tumor growth is sustained by a local increase of vascularization, leading to the proposal that vessel count is an independent prognosis marker of metastasis (1). This local hypervascularization is thought to result from the release by these tissues of angiogenic growth factors. Several studies have shown that vascular endothelial growth factor (VEGF) plays a major role in pathological neovascularization (2, 3). VEGF has been isolated from several tumor cell lines (4, 5, 6) and cloning of the gene provided evidence that at least five peptides of 121, 145, 165, 189 and 206 amino acids are generated by alternative splicing of the VEGF pre-mRNA (7, 8).

In nude mice, tumor progression of prostate cancer cell line xenografts decreases after anti-angiogenic treatments such as angiostatin (9), neutralizing antibodies against VEGF (10) or conversely is stimulated by activating VEGF transduction pathways (11). There is now some direct evidence that organ size and tissue mass are under the control of vascularization in adults. A recent report has shown that castration induces a parallel decrease of ventral lobe mass and its content in vascular endothelial cells (12). Testosterone injections restored endothelial cell content before the onset of prostate enlargment thus suggesting that potent prostatic angiogenic factors were up-regulated by androgen supply.

We designed this study to demonstrate whether prostatic expression of VEGF was controlled by androgens in physiological situations. We demonstrate that androgens up-regulate transiently VEGF expression at the mRNA and protein level in the normal human prostatic epithelial cell lines PNT1 without affecting the splicing of its mRNA. Furthermore testosterone injection in adult rats induced a moderate increase of tissue mass in the ventral lobe and a 4-fold increase in VEGF specific activity.

MATERIALS AND METHODS

Cell culture. The human epithelial cell line PNT1 (Dr O. Cussenot, Hopital Saint-Louis, Paris) has been immortalized by transfection with SV 40 large T antigen (13). These cells were cultured in DMEM medium supplemented with 100 Uml penicillin and streptomycin supplemented with 10% fetal calf serum and received 10^{-9} M DHT every other day. Prior to the onset of experiments the cell cultures were transfered to the DMEM medium supplemented without DHT for three days. 10^{-11} to 10^{-7} M DHT or 100 ng/ml human recombinant FGF1, PDGFAA, PDGFBB, EGF or 10 ng/ml FGF2 were added for different time intervals. The cells were rinsed with

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Abbreviations: DHT, dihydrotestosterone; PNT1, human immortalized epithelial prostatic cell line; VEGF, vascular endothelial growth factor.

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cold PBS and processed for RNA extraction. In parallel experiments confluent cell monolayers were rinsed 3 times and incubated with serum free medium supplemented with in the presence or absence of modulators (5). The conditioned media were collected 48 hours later.

Animals and treatments. Adult male Sprague-dawley rats (weight 280–300 g) received every three days an injection of 6 mg testosterone diluted in castor oil in order to provide a slow release of active hormone (14). At different time intervals (1, 3, 7 and 10 days) the prostates were dissected into ventral or laterodorsal lobes and immediately frozen in liquid nitrogen. Three animals were sacrificed for each point.

VEGF mRNA extraction and analysis. Confluent cells were lysed in guanidium thiocyanate and total RNA was purified by the phenol/chloroform method (15). Total RNA (20 μg) was run on a 1.2% formaldehyde agarose gel, blotted on to a Hybond-N nylon membrane (Amersham) by electrotransfer and UV-crosslinked. Hybridization was performed with a 605 bp BamH1 fragment of human VEGF cDNA (7) kindly provided by Dr J. Abraham (Scios Nova, Mountain View, CA). This probe was ^{32}P-labelled using a Megaprime DNA labelling system (Amersham). The control for equal lane loading and transfer was assessed by ethidium bromide staining. The gels were dried, analyzed by a phosphoimager and the relative amount of each band was assessed by image analysis.

Total RNA (2 μg) was used for the reverse transcriptase (RT) reaction using the Superscript preamplification system (GibcoBRL) and random hexamers as primers. Polymerase chain reaction (PCR) was performed on 1/10th of the cDNAs in a 50 μl volume using 2.5 units of Taq polymerase and 50 pmoles of each oligonucleotide flanking the 5′ and 3′ ends of the VEGF coding sequence (CAAGTACCAAAGCCTCC and ACTGTTCGGCTCCGCCACT). Amplification was performed for 30 cycles (94°C for 1 min; 57°C for 1 min; 72°C for 1.5 min). The amplified PCR products (6 μl) were separated by electrophoresis on a 1.5% agarose gel. Negative controls were performed in the absence of cDNA. The specificity of the amplification was authenticated by NcoI enzymatic restriction at the unique site located at position 151 (16). The cleavage products of the VEGF cDNAs were thereafter shorter by 86 bp.

VEGF measurement. Rat tissues were powdered under liquid nitrogen immersion and extracted in RIPA buffer supplemented with 10 μg/ml of antiproteases (aprotinin, leupeptin, pepstatin and benzamidin). After 30 minutes on ice, the extracts were centifuged and the supernatant frozen until use. VEGF content of the tissues and of the conditioned media was measured by radio receptor assay as already described (17). The results are expressed by comparison to a standard curve of human recombinant VEGF 165 amino acids diluted in the same vehicule (RIPA or culture medium respectively). Values are presented as means ± SEM of VEGF concentration per ml of conditioned medium or mg of wet weight prostate. The statistical analysis was performed by the test U of Mann and Whitney.

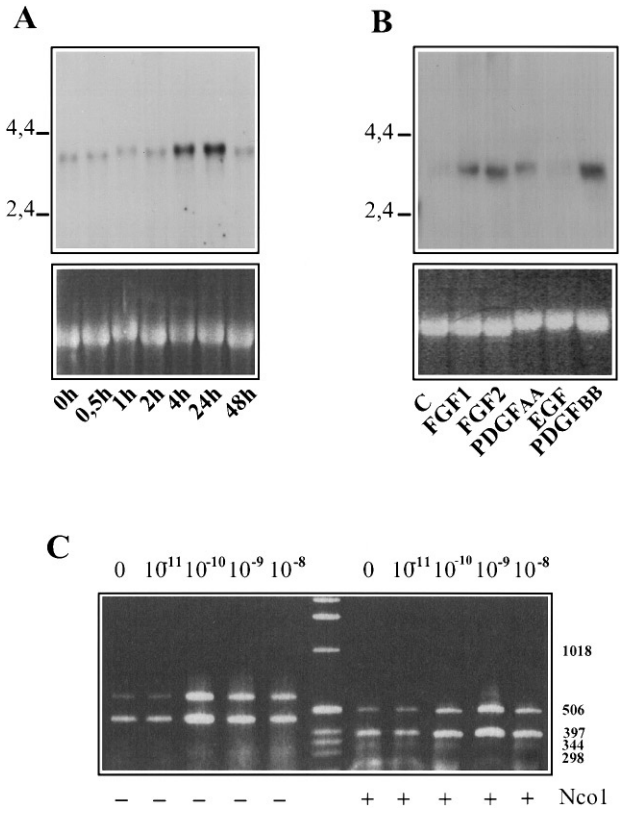

FIG. 1. VEGF mRNA expression in human PNT1 epithelial cells. PNT1 cells were exposed to 10^{-9} M DHT for various lenghts of time (A) or to various growth factors for 24 hours (B) and total RNA was extracted. 20μg was electrophoresed and hybridized with a ^{32}P-labelled human VEGF probe. Autoradiography was obtained by exposure to X-Omat AR film for 24h at −70°C. Position of VEGF mRNA is estimated by comigration with standard RNA marker (Gibco BRL). (C) RT-PCR analysis of VEGF, 2 μg of total RNA of PNT1 cells exposed for 24 hours to various concentrations of DHT was reverse transcribed and random single strand cDNAs were submitted to amplification using the primers described in the text, and visualized on a 1.5% agarose gel stained with ethidium bromide, size of PCR products was estimated by comigration with a standard molecular weight marker (1 kb ladder, BRL) deposited in the middle lane of the gel.

RESULTS

VEGF gene expression was measured by northern blot. VEGF mRNA increased rapidly after exposure of PNT1 cells to DHT. The maximal expression (3 fold) was observed after 4 hours, persisted up to 24 hours and decreased after 48 hours (Figure 1A). VEGF mRNA increased as a function of the dose between 10^{-11} (x1.3) and 10^{-9} (x2.1) and remained constant at concentrations of 10^{-8} (x1.9) and 10^{-7} (x1.8) M (data not shown). A similar increase in VEGF mRNA accumulation was observed upon exposure of PNT1 cells to FGF1, FGF2, PDGFAA or PDGFBB (1.8, 1.9, 1.5 and 1.9 fold greater than the unstimulated culture). Conversely EGF did not increase VEGF mRNA accumulation (Fig 1B).

To investigate the expression of the different VEGF isoforms, randomly primed first-strand cDNAs were amplified with human VEGF primers located in exons 1 and 8 to simultaneously amplify the different splice variants. Two bands (453 and 587 bp) corresponding to VEGF 121 and 165 were observed. NcoI extensive digestion of RT-PCR products generated 86 bp smaller fragments. Their relative intensity was unaffected by DHT treatment (Fig 1C).

The bioavailability of VEGF in PNT1 culture medium increased as a function of the dose of DHT (Fig 2A). Maximal stimulation was observed for 10^{-8} M DHT (x4.1) and remained constant for 10^{-7} M (x4.2). This dose-response curve shows that the increase of

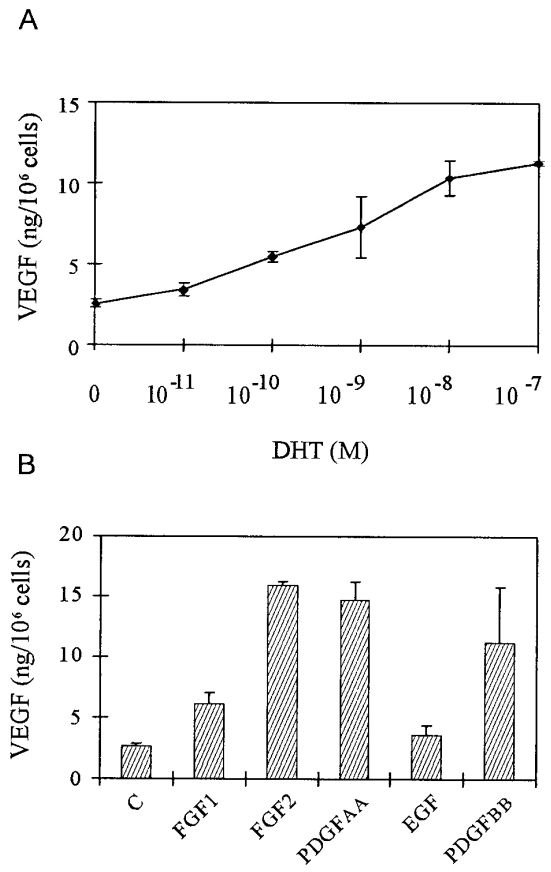

FIG. 2. VEGF secretion in PNT1 culture medium stimulated with DHT. Confluent monolayers of PNT1 cells cultured without DHT for 3 days were rinsed and exposed to various concentrations of DHT diluted in DMEM medium or 100 ng/ml FGF1, PDGFAA, PDGFBB, EGF or 10ng/ml FGF2. Two days later the conditioned medium was collected and the concentration of VEGF was measured by radio receptor assay. Results are expressed as the mean. of triplicate assays by comparison to a standard curve of recombinant VEGF 165 amino acids. Data are representative of 3 different experiments.

bioavailability requires doses of DHT an order of magnitude higher than that providing the maximal increase of VEGF mRNA. FGF2 and PDGFAA induced a similar stimulation of VEGF bioavailability (Fig 2B), whereas exposure to FGF1 or PDGFBB resulted in a moderate but significant increase.

Androgen treatment in adult rats resulted in a rapid increase of VEGF specific activity in the ventral lobe. Its concentration increased 2-fold on the first day following the onset of the treatment, 4-fold between day 3 and day 7 and then decreased (Figure 3). Similar changes, although less pronounced and more transient, were observed in laterodorsal lobe. A significant increase of the ventral lobe weight (x1.8) occured 3 days after testosterone injection. Therefore VEGF prostatic content is increased by 7-fold between days 3 and 6 post testosterone injection. Despite a transient 3-fold induction of VEGF specific activity in the laterodorsal lobe, its weight was not significantly changed.

DISCUSSION

The present study shows that prostatic expression of VEGF is up-regulated *in vitro* and *in vivo* by physiological concentrations of androgens.

Several normal cultured cells, such as smooth muscle cells (7), keratinocytes, epithelial (16) or fibroblastic cells (17) express VEGF at the mRNA and protein level. Although PNT1 cells are immortalized prostate epithelial cells, the expression of VEGF is unlikely due to the immortalization but rather reflects the level of VEGF expression observed in cultured normal epithelial cells. Accordingly we found that the tumoral prostatic cell lines PC3 and DU 145 secreted 4 to 6-fold more VEGF than PNT1 (data not shown). However it has been previously shown that the level of VEGF expression was only slightly increased in immortalized epithelial cells as compared to their normal counterparts (16). VEGF mRNA expression is known to be stimulated by several growth factors such as FGF2 (19), PDGF (20), TGF α (21), TGFβ (18) or TNFα (19).

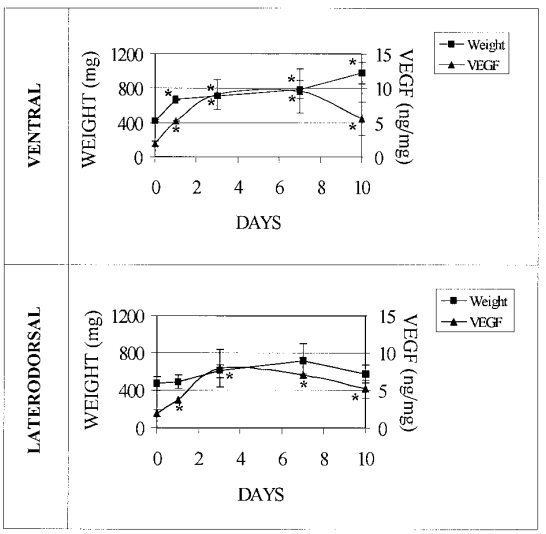

FIG. 3. Effects of testosterone on prostate weight and VEGF specific activity. Adult male Sprague-dawley rats (weight 280-300 g) received every three days an injection of 6 mg testosterone diluted in castor oil. At different time intervals the ventral and laterodorsal lobes of the prostates were dissected and immediately frozen in liquid nitrogen. The tissues were powdered under liquid nitrogen immersion and extracted in RIPA buffer supplemented with 10 μg/ml of antiproteases (aprotinin, leupeptin, pepstatin and benzamidin). After 30 minutes on ice, the extracts were centifuged and VEGF content was measured by radio receptor assay in the supernatants. 3 animals were used for each point. The results are expressed by comparison of a standard curve of human recombinant VEGF 165 amino acids. Values are presented as means $\pm$ SEM. The statistical analysis was performed by the test U of Mann and Whitney. (*) $p<0.05$.

Accordingly VEGF expression was up regulated by FGF2, FGF1, PDGFAA, PDGFBB in PNT1. Although EGF stimulates VEGF production in glioblastoma cells (22) it has no action on PNT1 cells. The mechanisms of VEGF up regulation by growth factors appear to involve interactions of transcription factors with the Sp1, AP1, AP2 sequences found in the VEGF promoter (7).

Although the VEGF promoter does not contain classical consensus androgen response sequences, we found that VEGF expression is up-regulated by DHT as a function of the dose at a level comparable to that obtained upon exposure to growth factors. RT-PCR analysis demonstrated that both the 121 and 165 amino acids isoforms encoded by alternative splice variants were expressed in PNT1 cells and that DHT did not seem to modify their relative expression. VEGF mRNA were actively translated in prostate epithelial cells in bioactive VEGF.

The physiological role of VEGF in adult vessels remains to be elucidated.

Androgen receptors are observed in epithelial and stromal cells of the ventral prostate (23). It was then tempting to examine the effects *in vivo* of androgens on VEGF expression. In fact testosterone injections increased VEGF expression in rat ventral prostate, and to a lesser extent in the other lobes. This effect appeared the day following the treatment and reached a maximum level within three days, corresponding to a 4-fold increase of the local bioavailability of VEGF in the ventral prostate. A transient increase of ventral lobe weight was observed between the third and the tenth day following androgen injections. However, despite an almost comparable increase of VEGF bioavailability in the laterodorsal lobe, its weight remained unchanged, suggesting that VEGF up-regulation is not sufficient to trigger an efficient increase in tissue mass. This discrepancy suggests that in addition to VEGF overexpression, other events, such as up-regulation of VEGF receptors or inhibition of the expression of endogenous VEGF receptor antagonists, are required to promote endothelial cell proliferation. Their expression might be differently regulated in the ventral and the laterodorsal lobes of the rat prostate. Further studies are required to confirm that VEGF overexpression in the ventral lobe of the prostate induces an increase of endothelial cell proliferation.

In summary, the present study demonstrates that VEGF expression in the prostate is regulated by androgens. A recent report has demonstrated that prostate endothelial cells proliferate after androgen injection in castrated rats (12) and has suggested that angiogenesis controls prostate regrowth after castration (24). These results document this hypothesis and suggest that the angiogenesis observed in prostate regrowth upon testosterone injection might be mediated by VEGF-VEGF receptors system.

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