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Vascular Endothelial Growth Factor Induces IP-10 Chemokine Expression

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We have previously shown that intracavernous injection of vascular endothelial growth factor (VEGF) improved the recovery of erectile function in an arteriogenic impotence rat mode. We wished to identify genes that are affected by VEGF treatment in the penis. Specifically we examined the induction of IP-10 chemokine. Male rats were subjected to pudendal arterial ligation or sham operation. They were then treated with intracavernous injection of 4 μg of VEGF in phosphate-buffered saline (PBS) or PBS alone. At 6 and 24 h after treatment, the erectile tissue was then harvested for RNA isolation. The isolated RNA was used for microarray and RT-PCR analyses. Cultured rat cavernous smooth muscle cells (CSMC) were treated with VEGF and then subjected to RT-PCR analysis. Cultured human CSMC were treated with VEGF and then subjected to ELISA analysis. Microarray analysis detected IP-10 as an abundantly induced message in 6-h VEGF-treated tissues. This was further confirmed by RT-PCR analysis. Using cultured rat CSMC, induction of IP-10 mRNA was detectable in 1 and 2 h, but not 24 h, VEGF-treated cells. Induction of IP-10 at the protein level was observed with cultured human CSMC. Secretion of IP-10 into culture medium peaked at 4 h after treatment of human CSMC with 10 ng/ml of VEGF. Optimal VEGF dosage for IP-10 induction was 50 ng/ml when assayed with cells that were treated for 8 h. © 2002 Elsevier Science (USA)

Key Words: **VEGF; IP-10; penis; smooth muscle; chemokine; angiogenesis.**

Angiogenesis is the formation of new blood vessels from preexisting microvasculature and is critical to many physiological and pathological processes. The regulation of angiogenesis is balanced between angiogenic and angiostatic factors that promote and inhibit neovascularization, respectively. An imbalance in favor of angiogenic factors can contribute to the pathogenesis of tumor growth and metastasis as well as the promotion of chronic fibroproliferative disorders.

The CXC chemokines are a subfamily of the chemokine superfamily and is comprised of members that possess either angiogenic or angiostatic activities [1]. CXC chemokines are heparin-binding proteins that have four highly conserved cysteine residues, with the first two separated by one non-conserved amino acid residue (therefore C-X-C). While the CXC motif distinguishes this family from other chemokine families, the presence or absence of another structural domain, the ELR motif (single-letter code for Glu-Leu-Arg), which precedes the first cysteine, further divides the CXC chemokines into those that are ELR^+ and those ELR^-. ELR^+ CXC chemokines promote angiogenesis and include interleukin-8 (IL-8), growth-related genes (GRO-α, -β and -γ), epithelial neutrophil-activating protein-78 (ENA-78), and granulocyte chemotactic protein-2 (GCP-2). ELR^- CXC chemokines inhibit angiogenesis and include platelet factor-4 (PF4), monokine induced by interferon-γ (MIG), and interferon-inducible protein-10 (IP-10).

IP-10 was first recognized as an early response transcript in interferon-γ-stimulated human U937 lymphoma cells [2]. The murine and rat homologs of IP-10 (60% nucleotide homology) were subsequently cloned and termed crg-2 [3, 4] and mob-1 [5], respectively. Constitutive expression of IP-10 (used as synonym for crg-2 and mob-1 throughout this report) has been observed in naïve thymus, spleen, lymph node, liver, and kidney. Inducible expression of IP-10 has been observed in a wide variety of tissues and cells under the influence of stimuli such as interferons, interleukins, lipopolysaccharide, tumor necrosis factor-α, platelet-derived growth factor, and hypoxia [6].

The principal biological role of IP-10 appears to be modulation of leukocyte development and function [6]. IP-10 has also been shown to stimulate proliferation and migration of vascular smooth muscle cells [7]. Other studies demonstrated that IP-10 was able to

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inhibit IL-8 and bFGF-induced neovascularization [8, 9] and inhibits endothelial cell proliferation [10]. The angiostatic potential of IP-10 has been exploited in cancer therapy trials with encouraging results [11, 12].

To encourage neovascularization in patients with ischemic conditions, the well-known angiogenic factor, vascular endothelial growth factor (VEGF), has been successfully employed in many animal and clinical studies. We have recently demonstrated that injection of VEGF into the corpus cavernosum was able to preserve the erectile function in an arteriogenic impotence rat model [13]. In addition to histological and electron microscopic examination of the VEGF-treated erectile tissues, a gene microarray analysis was carried out in an attempt to identify possible gene expression changes. We report here that one of the most highly expressed genes following VEGF treatment was IP-10. Increase of the induced IP-10 mRNA and protein expression in a dose- and time-dependent manner was further demonstrated in cultured cavernous smooth muscle cells treated with VEGF.

MATERIALS AND METHODS

Rat model and VEGF injection. The experimental protocol and animal care were approved by our institutional Committee on Animal Research. Male Sprague–Dawley rats of 6 months of age were used in all experiments. Arteriogenic insufficiency rat model with pudendal artery ligation was established as previously described [14]; intracavernous injection of VEGF (4 μg/injection/rat) was also performed as previously described [13]. Four groups of rats (6 rats per group) were established: Sham + PBS, Sham + VEGF, Ligation + PBS, and Ligation + VEGF. Three rats in each group were treated with PBS or VEGF and sacrificed 6 hours later and the remaining 3 rats in each group were treated similarly and sacrificed at 24 h. At the time of sacrifice (6 or 24 h), electrostimulation of the cavernous nerve was performed and intracavernous pressure (ICP) measured. All sham-operated rats had normal ICP of ~100 cm H_2O and all ligated rats (with or without VEGF treatment) had ICP of ~30 cm H_2O. The erectile tissue was then processed for RNA isolation.

RNA preparation. RNAs of cavernous tissues and cell cultures were isolated by the Tri-Reagent RNA extraction method (Molecular Research Center, Cincinnati, OH). Despite the manufacturer's claim, RNAs prepared by this method usually are contaminated by trace amount of DNA, as assessed by polymerase chain reaction (PCR) with β-actin primers (data not shown). To remove the contaminating DNA, each RNA sample (20–50 μg) was treated with 10 units of RNase-free DNase I (Roche Molecular Biochemicals, Pleasanton, CA) at 37°C for 30 min. The RNA was then purified by phenol/chloroform extraction and ethanol precipitation. Quantity and purity of RNAs were measured by spectrophotometry with UV adsorption at wavelengths 260 and 280. Their integrity was visualized by the sharpness of the 28S and 18S ribosomal RNA bands in agarose gels.

Microarray analysis. Tissue RNA was converted into ^{32}P-labeled cDNA probe, which was then hybridized to cDNA fragments of 1176 rat genes that have been immobilized on a nylon membrane (Atlas Rat 1.2 Array, Clontech Inc., Palo Alto, CA). The detail of this procedure has been described previously [14].

Oligonucleotide primers. Primer pairs for RT-PCR analysis of IP-10 and β-actin genes are listed in Table 1.

TABLE 1

Oligonucleotide Primers

Gene	Primer name	Sequence	Size of PCR product
β-Actin	β-actin-s	TCTACAATGAGCTGCGTGTG	
	β-actin-a	ATCTCCTTCTGCATCCTGTC	682 bp
IP-10	IP-10-s	AAGCACCATGAACCCAAGTG	
	IP-10-a	CTCTCTAGTTACGGAGCTCT	312 bp

RT-PCR analysis. RT-PCR (reverse transcription-polymerase chain reaction) was performed in an RT step and a PCR step, as described previously [14]. Briefly, 2.5 μg of each RNA was reverse transcribed in a reaction volume of 20 μl. This RT product was then diluted 5- to 100-fold with TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA). One microliter of each dilution was used in a 10 μl PCR to identify the optimal input within the linear amplification range. PCR was performed in DNA Engine thermocycler (MJ Research, Inc., Watertown, MA) under calculated temperature control. The cycling program was set for 35 cycles of 94°C, 10 s; 55°C, 10 s; 72°C, 10 s, followed by one cycle of 72°C, 5 min. The PCR products were electrophoresed in 1.5% agarose gels in the presence of ethidium bromide, visualized by UV fluorescence, and recorded by a digital camera connected to a computer.

Rat cell culture and VEGF treatment. Rat cavernous smooth muscle cells (CSMC) were cultured as previously described [15]. Cells were seeded at 4×10^5 cells per well in 3 ml of DMEM with 10% FBS in 6-well culture plates. In 48 to 72 h, the cells reached ~80% confluence and were then treated with 12.5 ng/ml VEGF (or PBS) for 1, 2, and 24 h. The cells were then harvested for RNA isolation, followed by RT-PCR analysis.

Quantification of IP-10. An ELISA kit for the quantification of IP-10 was purchased from R&D Systems (Minneapolis, MN). The kit is specific for human IP-10; therefore, human cavernous smooth muscle cells, isolated from a man of normal erectile function [16], were used in the experiments. Cells were seeded at 5×10^4 cells per well in one ml of DMEM with 10% FBS in 12-well culture plates. In 48 to 72 h, the cells reached ~80% confluence and were then treated with VEGF for time-course and dose–response measurements. For time-course experiments, cells were treated with 10 ng/ml VEGF and the culture medium was harvested at 0, 0.5, 1, 2, 4, 8, 24, and 48 h. For dose–response experiments, cells were treated with VEGF at 0, 0.1, 1, 10, 25, 50, and 100 ng/ml and the medium was harvested 8 h later. All assays were performed in triplicate in each experiment and all data presented under Results are the average of three independent experiments.

RESULTS

Microarray analysis. Microarray analysis identified several genes that showed either higher or lower expression levels among different treatment groups of rats. In this report, we will only focus on the IP-10 gene, whose expression was undetectable in the corpus cavernosum of normal, sham-operated, or PBS-treated rats, but was highly induced in VEGF-treated rats (Fig. 1).

RT-PCR analysis. To verify the microarray analysis results, rat tissue RNAs were further analyzed by RT-PCR. As shown in Fig. 2, IP-10 expression was detectable only in tissues that were treated with

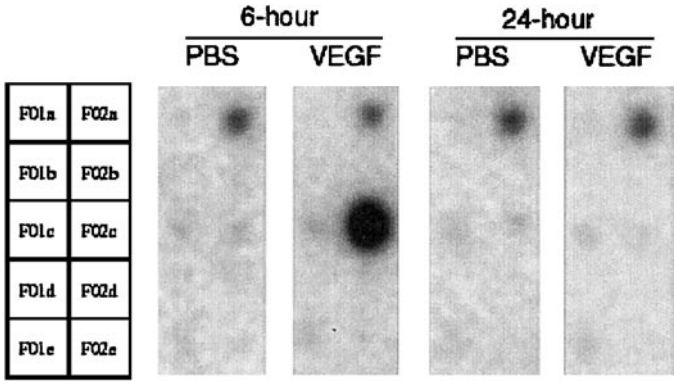

FIG. 1. Identification of IP-10 induction in penile tissues by microarray analysis. Rats were subjected to intracavernous injection of PBS or VEGF. After 6 or 24 h, their erectile tissues were collected and subjected to microarray analysis. The resulting autoradiographs are partially shown in this figure, focusing on IP-10 and its neighboring 9 genes, the identity of which can be found in the Rat12.xls document of Clontech (http://www.clontech.com/atlas/genelists/index.html) using the coordinates provided on the left (F01a to F02e). F02c is IP-10.

VEGF, regardless of the type of surgical procedures. The induction appeared to be short-lived, as 24-h VEGF-treated samples showed very little expression when compared with the 6-h VEGF-treated samples.

IP-10 mRNA induction in cultured cells. To determine whether induction of IP-10 could be reproduced in cell culture, we performed experiments using cultured rat CSMC, which express VEGFR1 receptor, as we previously described [15]. Treatment of CSMC with 12.5 ng/ml of VEGF for 1 and 2 h resulted in IP-10 mRNA expression. Twenty-four hours after treatment, CSMC no longer expressed IP-10 (Fig. 3).

IP-10 protein induction in cultured cells. To further verify IP-10 induction at the protein level and to establish its time course and dose-dependence, we performed ELISA with cultured human CSMC. As shown

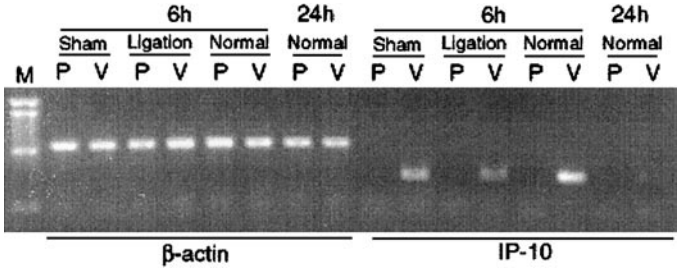

FIG. 2. Identification of IP-10 induction in penile tissues by RT-PCR analysis. Rats were subjected to sham-operation (Sham), pudendal arterial ligation (Ligation), or not operated (Normal) and then treated with intracavernous injection of PBS (P) or VEGF (V). After 6 or 24 h, their erectile tissues were collected and subjected to RT-PCR analysis for IP-10 and β-actin expression. Lane M is 100-bp size marker.

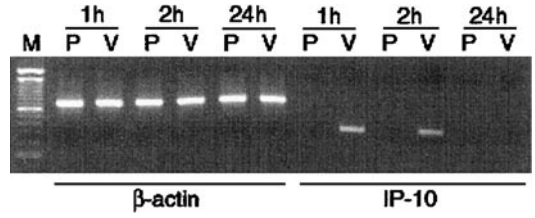

FIG. 3. Identification of IP-10 induction in cultured rat CSMC. Cells were treated with 12.5 ng/ml VEGF (V) or PBS (P). At hour 1, 2, and 24, they were harvested for RT-PCR analysis for IP-10 and β-actin expression. Lane M is 100-bp size marker.

in Fig. 4A, secretion of IP-10 peaked at 4 h after cells were treated with 10 ng/ml of VEGF. The VEGF dosage that induced the highest level of secreted IP-10 was 50 ng/ml when assayed with cells that were treated with VEGF for 8 h (Fig. 4B).

DISCUSSION

VEGF is the most intensely studied angiogenic growth factor. Both its beneficial effects in treating

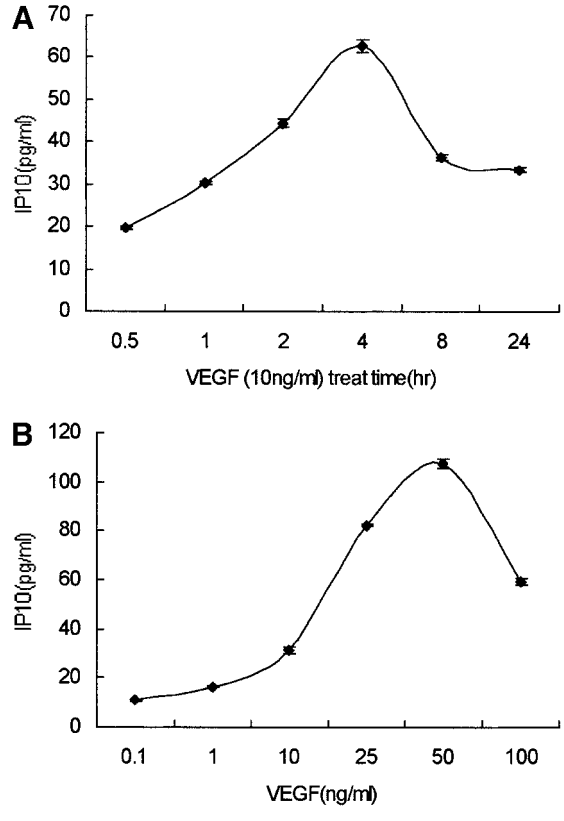

FIG. 4. Time course (A) and dose response (B) for expression of IP-10 in VEGF-treated human CSMC. Human CSMC were treated with a fixed dose (12.5 ng/ml) VEGF for the indicated periods of time (A), or, treated for a fixed period of time (8 h) with the indicated dosages of VEGF (B). Their culture media were then subjected to ELISA analyses.

ischemic conditions and its underlying threat in promoting tumor growth have been extensively documented. In an arteriogenic impotence model, we have shown that VEGF was able to improve erectile function with concomitant preservation of penile nerve and smooth muscle structures [13]. To investigate the underlying molecular changes, we examined the VEGF-treated tissues by the microarray procedure. In this report we demonstrated the induction of IP-10 as one of the most profound gene expression changes associated with VEGF treatment.

IP-10 has been known as an inducible chemokine in a variety of cells responding to stimuli such as interferons and interleukins. To our best knowledge, this report is the first to demonstrate that IP-10 could be induced by VEGF. More importantly, our findings raise a previously unknown aspect of the neovasculogenic pathways—induction of an angiostatic factor (IP-10) by an angiogenic factor (VEGF).

Induction of IP-10 by VEGF appears to be rapid but short-lived. The quick response implies an urgent need for the stimulated cells to counter an oversupply of VEGF. Particularly, the stimulated cells in this case were CSMC, which are likely the principal source for VEGF production in the corpus cavernosum [15]. Since VEGF is normally expressed at low levels in most tissues and cells, the sudden rise of VEGF concentration as a result of intracavernous VEGF injection (or addition of VEGF to culture medium) could possibly be sensed by CSMC (which possess VEGFR1 receptor) as a threat that could lead to excessive neovascularization. To counteract such a threat, gene expression of IP-10, a known angiogenic factor, was therefore rapidly turned up. However, even with the implementation of such a protective mechanism, an eventual balance in favor of neovascularization would nevertheless take place, as many studies, including ours, have shown that injection of VEGF indeed results in formation of new blood vessels. As such, the exact physiological role of an elevated but short-lived IP-10 expression as a response to VEGF stimulation remains to be determined.

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