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Tumor Necrosis Factor-α Regulates Expression of Vascular Endothelial Growth Factor Receptor-2 and of Its Co-receptor Neuropilin-1 in Human Vascular Endothelial Cells*

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Tumor necrosis factor-α (TNF-α) modulates gene expression in endothelial cells and is angiogenic *in vivo*. TNF-α does not activate *in vitro* migration and proliferation of endothelium, and its angiogenic activity is elicited by synthesis of direct angiogenic inducers or of proteases. Here, we show that TNF-α up-regulates in a dose- and time-dependent manner the expression and the function of vascular endothelial growth factor receptor-2 (VEGFR-2) as well as the expression of its co-receptor neuropilin-1 in human endothelium. As inferred by nuclear run-on assay and transient expression of VEGFR-2 promoter-based reporter gene construct, the cytokine increased the transcription of the VEGFR-2 gene. Mithramycin, an inhibitor of binding of nuclear transcription factor Sp1 to the promoter consensus sequence, blocked activation of VEGFR-2, suggesting that the up-regulation of the receptor required Sp1 binding sites. TNF-α increased the cellular amounts of VEGFR-2 protein and tripled the high affinity ^{125}I-VEGF-A$_{165}$ capacity without affecting the K_d of ligand-receptor interaction. As a consequence, TNF-α enhanced the migration and the wound healing triggered by VEGF-A$_{165}$. Since VEGFR-2 mediates angiogenic signals in endothelium, our data indicate that its up-regulation is another mechanism by which TNF-α is angiogenic and may provide insight into the mechanism of neovascularization as occurs in TNF-α-mediated pathological settings.

A well regulated angiogenesis is critical for embryonic growth, bone remodeling, menstrual cycle, corpus luteum formation, and tissue repair. The stable vascular bed occurring in these physiologic conditions results from a balance of signals that favor angiogenesis and those that promote vascular regression. In contrast, a deregulated angiogenesis is pivotal in tumor progression and inflammatory and viral diseases (1–3). A number of naturally occurring growth factors can directly induce angiogenesis by stimulating endothelial cell proliferation and migration or act indirectly by triggering endothelial cells themselves or accessory cells (monocyte/macrophage, mastocytes, T cells) to release direct angiogenic inducers (1–3).

Tumor necrosis factor-α (TNF-α)[1] is a powerful activator of angiogenesis *in vivo* in several animal models when used at low doses (4–6) but is inhibitory at high doses (7). However, the ability of TNF-α to induce *in vitro* biological responses related to angiogenesis is weak. TNF-α stimulates *in vitro* chemotaxis of bovine adrenal capillary endothelial cells (4) but inhibits wound repair (8) and is devoid of mitogenic activity (5). Angiogenesis promoted by TNF-α seems necessarily to be due to indirect effects. TNF-α activates in endothelial cells the synthesis of B61 (9), basic fibroblast growth factor (FGF) (10), and platelet-activating factor (11), all known to be angiogenic (6, 12), and of tissue factor (13), which is a regulator of vessel formation (14). In endothelial cells, TNF-α promotes the synthesis of urokinase-type plasminogen activator (15), which is involved in the progression phase of angiogenesis characterized by a remodeling of extracellular matrix proteins by proteolytic enzymes (Ref. 16; for reviews, see Refs. 1–3).

TNF-α cooperates with basic FGF, vascular endothelial growth factor-A (VEGF-A), and interleukin-8 to induce capillary-like tubular structure of human microvascular endothelial cell growth in a three-dimensional gel of extracellular matrix proteins (17, 18). In these systems, the type of extracellular matrix seems to address the features of the angiogenic model. TNF-α does not induce angiogenesis *in vitro* when the cells are plated on three-dimensional fibrin matrix, but it is permissive for the activity of basic FGF and VEGF-A. TNF-α up-regulates the activity of urokinase-type plasminogen activator, which is required for the formation of capillary structure in addition to the angiogenic molecules (17). Otherwise, TNF-α induces *in vitro* angiogenesis of endothelium plated on collagen type I. This activity is mediated by the release of VEGF-A, basic FGF, and interleukin-8 (18). Furthermore, TNF-α induces mesenchy-

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[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; BSA, bovine serum albumin; FCS, fetal calf serum; FGF, fibroblast growth factor; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; kb, kilobase pair(s); MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

mal or tumor cells to release angiogenic molecules, including VEGF-A (7, 19). Finally, it has been reported that TNF-α regulates the expression of integrins involved in adhesion of endothelial cells to extracellular matrix and in angiogenesis (8, 20).

The puzzling effects of TNF-α on endothelial cells and new vessel growth suggest the presence of more than one angiogenic signaling pathway and that this cytokine may have different activities on endothelial cells depending on the microenvironment. In light of the relevance of the cooperation between TNF-α and VEGF-A (7, 17, 18) in angiogenesis, we studied the effect of TNF-α on the expression and function of VEGF receptors. Adult endothelial cells express on their surface VEGF receptor (VEGFR)-1 encoded by *Flt-1* (21) and VEGFR-2 by *KDR/Flk-1* (22, 23), but recent findings suggest that the latter alone is able to mediate the mitogenic and chemotactic effect of VEGF-A in endothelial cells (22, 24, 25). More recently, it has been reported that neuropilin-1, a receptor that mediates neuronal cell guidance (26), is expressed by endothelial cells and enhances the binding of VEGF-A_{165} isoform to VEGFR-2 (27). Here, we demonstrate that the pretreatment of endothelial cells with TNF-α is followed by an increased migration and wound repair induced by VEGF-A_{165}. An augmented expression of VEGFR-2 and neuropilin-1 genes causes this effect.

EXPERIMENTAL PROCEDURES

Cell Cultures—Human umbilical vein endothelial cells, prepared and characterized as described previously (28), were growth in medium 199 (Life Technologies, Inc.) supplemented with 20% fetal calf serum (FCS) (Irvine, Santa Ana, CA), endothelial cell growth supplement (100 μg/ml), porcine heparin (50 units/ml), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (all from Sigma), in gelatin (Life Technologies, Inc.)-coated tissue culture plates (Falcon, Becton Dickinson, Plymouth, UK). They were used at early passages (I–III). Human fibrosarcoma 8378 cells, which respond to TNF-α (29), were maintained in Dulbecco's modified Eagle's medium containing 10% FCS. Porcine aortic endothelial cells transfected with human VEGFR-2 (24) were cultured in Ham's F-12 (Sigma) supplemented with 10% FCS. Human foreskin microvascular endothelial cells isolated as described previously (17), were cultured on fibronectin-coated dishes in medium 199 buffered with 20 mM Hepes containing 10% human serum, 10% newborn calf serum, endothelial cell growth supplement (150 μg/ml), and porcine heparin (5 units/ml).

Experimental Design—To verify the effects of TNF-α on the expression of VEGF receptors and on the biological activities elicited by VEGF-A_{165}, the following experimental conditions have been used: confluent endothelial cell growth at a CO_2 level of 5% in atmospheric air was treated with TNF-α (1×10^7 units/mg of protein; Genentech, Inc., San Francisco, CA) in medium 199 supplemented with 20, 5, and 1% FCS or 1% bovine serum albumin (BSA) (lipopolysaccharide-free, Sigma), twice washed with medium 199, and then used to extract RNA. Alternatively, cells were stimulated with VEGF-A_{165} (a gift of Dr. H. A. Weich, GBF, Braunschweig, Germany) (30) in medium 199 containing 1% FCS in chemotaxis or 3% BSA in wound healing experiments. In some experiments, endothelial cells were starved for 24 h in medium 199 containing 1% FCS and 1% BSA before adding TNF-α. The effect of mithramycin (Sigma), which inhibits gene expression by blocking Sp1 binding to the CG box (7, 31), was studied by treating the cells for 12 h in medium 199 containing 5% FCS with or without TNF-α.

RNA Extraction and Northern Analysis—Total cellular RNA was isolated by guanidinium isothiocyanate extraction and centrifugation through cesium chloride (32). Equal amounts of total RNA (15 μg/lane) were electrophoresed in 1% agarose gels containing 6.3% formaldehyde in MOPS buffer (Sigma) and blotted on a Nylon Duralon-UV membrane (Stratagene) by the traditional capillary system in 10× SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7) (32). Filters were cross-linked with UV light (0.5 J/cm^2) and prehybridized for 4 h at 42 °C in 50% formamide deioinizate, 10% dextran sulfate, 1% SDS, 1 M NaCl, and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out overnight at 42 °C with [α-^{32}P]dCTP-labeled (3000 Ci/mmol, Amersham, Buckinghamshire, United Kingdom, UK) human VEGFR-2 (a 0.729-kb *Hin*dIII–*Eco*RI of KDR cDNA) (23), VEGFR-1 (a 1.347-kb *Hin*dIII–*Bgl*II fragment of human FLT-1 cDNA) (21), neuropilin-1 (a 0.735-kb *Pst*I–*Pst*I fragment of human neuropilin-1 cDNA) (27) and β-actin cDNAs (33). cDNAs were labeled using Rediprime random primer labeling kit (Amersham) according to manufacturer's instructions. Posthybridization washes were performed at high stringency (once in 2× SSC, 0.1% SDS for 30 min, once in 0.2× SSC, 0.1% SDS for 30 min and twice in 0.1× SSC, 0.1% SDS for 30 min) at 57 °C, and the membranes were exposed on autoradiography with Hyperfilm-MP (Amersham).

Nuclear Run-on—Nuclei were isolated from cultured endothelial cells essentially according to Ref. 34. Briefly, cells (2×10^7 cells/assay) were washed twice with ice-cold phosphate-buffered saline (PBS), scraped and collected in a 15-ml centrifuge tube by centrifugation at $500 \times g$ for 5 min at 4 °C. Subsequent steps were performed at 4 °C. The cells were resuspended in 4 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40) and allowed to stand on ice for 5 min. and then centrifuged at $500 \times g$ at 4 °C for 5 min. Nuclei were resuspended in 200 μl of glycerol storage buffer (10 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and frozen in liquid N_2. *In vitro* transcription and isolation of the resulting nuclear RNA were performed as described by Ikeda *et al.* (35). Two-hundred μl of frozen nuclei were thawed and mixed with 200 μl of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl 10 mM dithiothreitol, 400 units/ml placental ribonuclease inhibitor (Stratagene), 20 mM creatine phosphate (Sigma), 200 μg/ml creatine phosphokinase (Sigma), a 1 mM concentration each of ATP, CTP, and GTP (Stratagene), and 100 μCi of [α-^{32}P]UTP (3000 Ci/mmol, Amersham). Samples were incubated at 30 °C for 30 min with shaking and for 5 min in the presence of 20 units of DNase I (RNase-free, Life Technologies, Inc.). After the addition of proteinase K (150 μg/ml, Sigma) and SDS (0.5% final concentration), incubation was continued at 37 °C for 30 min. Extracted RNA was resuspended in TES buffer (10 mM TES, pH 7.4, 10 mM EDTA, 0.2% SDS) at 5×10^6 cpm/ml. Linearized plasmids containing the target cDNAs (15 μg) were immobilized onto a nylon Duralon-UV membrane (Stratagene) using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The filters were prehybridized overnight at 42 °C with hybridization buffer containing 20 mM PIPES (Sigma), pH 6.4, 50% formamide (Sigma), 2 mM EDTA, 0.8 M NaCl, 0.2% SDS, 1× Denhardt's solution (0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone), 200 μg/ml *E. coli* tRNA (RNase-free, Stratagene). Hybridization was at 42 °C for 48 h in the same solution supplemented with 15×10^6 total cpm of labeled RNA. The filters were washed twice in 2× SSC, 0.5% SDS at 42 °C for 30 min, twice in 0.3× SSC, 0.5% SDS, at 42 °C for 30 min and then incubated with 10 μg/ml RNase A in 2× SSC at 37 °C for 30 min. Further washed were done in 2× SSC at 37 °C for 30 min and then in 0.3× SSC at 37 °C for 30 min. The filters were exposed on autoradiography with Hyperfilm-MP and intensifying screens at −80 °C. The amount of VEGFR-2 mRNA was standardized by comparison with the amount of β-actin mRNA. Densitometric analysis was performed with a GS250 Molecular Imager (Bio-Rad).

Western Blot—Endothelial cells were washed twice with PBS and lysed on ice with 1 ml of 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 5 mM EDTA, 50 μg/ml pepstatin, 100 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 500 μg/ml soybean trypsin inhibitor (all from Sigma). After centrifugation (20 min at 4 °C at $13,000 \times g$), protein were solubilized, separated by SDS-polyacrylamide gel electrophoresis (7%), transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore Corp., Bedford, MA), probed with rabbit anti-VEGFR-2 antibody (C-1158, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and detected by ECL (Amersham).

Binding Assay and Analysis—Recombinant VEGF-A_{165} (2 μg) was dissolved in 200 μl of sodium phosphate buffer 20 mM, pH 7.4, and transferred in IODO-GEN-coated tubes (50 μg/ml) (Pierce), where VEGF-A_{165} was iodinated (5 min, 4 °C) with 1 mCi of ^{125}I (Amersham). Twenty μl of phosphate buffer 20 mM, pH 7.2, containing 1% BSA, 0.4 M NaCl, 0.1% CHAPS (Pierce) was added, and the reaction products were separated on Sephadex-G10. The specific activity of the tracer was 90,000 cpm/ng. ^{125}I-VEGF-A_{165} retained its biological activity as measured by migration of endothelial cells (28). For specific binding studies confluent cells plated in 24-well plates were incubated an orbital shaker at 4 °C for 2 h in 200 μl/well of binding medium (medium 199 containing 20 mM Hepes buffer, pH 7.4, 0.1% BSA, 100 μg/ml soybean trypsin inhibitor) with increasing concentrations of ^{125}I-VEGF-A_{165} in the presence of a 100-fold excess of unlabeled ligand. Endothelial cells were washed three times with ice-cold PBS containing 0.1% BSA and lysed in 200 μl/well of SDS 2% in PBS. Lysates were counted using a Beckman γ 5500B counter. Triplicate samples under each condition were obtained for each experiment. Specific binding, calculated subtracting from the total cpm bound after incubation with a 100-fold excess of unlabeled ligand, was approximately 80%. The K_d was esti-

mated by Scatchard plot using the Ligand program (Elseviere-Biosoft, Cambridge, UK).

Transient Transfection of pGL2basicFLK Plasmid—The 2.0-kb *Xho*I/*Sac*I fragment of *Flk-1* promoter (36) was subcloned in pGL2basic plasmid (Promega, Madison, WI) to generate the luciferase reporter vector pGL2basicFlk-1.[2] Human fibrosarcoma 8387 cell line (2×10^5 cells/well) was transfected with 3 μg of pGL2basicFlk-1 or pGL2basic using Superfect Transfection Reagent according to the manufacturer's instructions (Qiagen, Inc., Valencia, CA).The generated plasmid-liposome complex in 0.6 ml of Dulbecco's modified Eagle medium containing 10% FCS was incubated with the cells for 3 h at 37 °C in 5% CO_2. The medium was then replaced with fresh medium, and the cells were stimulated for 4 h with TNF-α (20 ng/ml) or left untreated. pSVgal construct (2.5 μg) was co-transfected to correct for the variability in transfection efficiency, and β-galactosidase activity was assayed with chlorophenol red β-D-galactopyranoside (Boehringer Mannheim GmbH, Mannheim, Germany) as a substrate. For final luciferase assay, cells were lysed in 0.2 ml of passive lysis buffer (dual luciferase assay, Promega) at 4 °C, and 20 μl of cleared (12,000 $\times$ *g* for 2 min at 4 °C) cell extract containing 50 μg of protein were mixed with 0.1 ml of luciferase assay buffer. Light production was measured for 5 s in a luminometer (Magic Lite Analyzer, Ciba Corning, Milano, Italy), and results were normalized to the β-galactosidase activity.

Migration Assay—Migration assay was performed as described previously with Boyden's chamber technique (28). Polycarbonate filters (5-μm pore size polyvinylpyrrolidone-free; Neuroprobe, Pleasanton, CA) were coated with 0.1% gelatin for 6 h at room temperature. VEGF-A_{165} in medium 199 supplemented with 1% FCS was seeded in the lower compartment of the chamber, and 1.25×10^5 resuspended cells in 50 μl of medium 199 containing 1% FCS were then seeded in the upper compartment. At the end of the incubation (37 °C in air with 5% CO_2 for 6 h), filters were removed and stained with Diff-Quik (Baxter Spa, Rome, Italy), and 10 high power oil immersion fields were counted. The results obtained were analyzed by one-way analysis of variance and the Student-Newman-Keuls test (Statistic Software; Bio-Soft).

Wound Healing—Human endothelial cells were grown at confluence on 24 wells and monolayers were treated for 24 h with TNF-α (10 ng/ml) or vehicle alone in medium 199 containing 5% FCS. After washes, the monolayer was wounded with a razor blade (lesion surface: 20 mm^2) as described (28) and incubated in medium 199 containing 3% BSA with or without VEGF-A_{165}. After 24 h, the cells were fixed and stained as described (28). To quantify the repair process, phase-contrast microscopic pictures of wounded monolayer were recorded with a still video camera recorder (R5000H; Fuji Photo Film Co., Tokyo, Japan), and cell number was counted in 10 fields of 1 mm^2 randomly selected, with a Cosmozone image analyzer (Nikon, Tokyo, Japan).

RESULTS

Increase of VEGFR-2 and Neuropilin-1 mRNA in Endothelial Cells Challenged with TNF-α—Several studies have shown that 7.0-kb VEGFR-2 (23, 37) and 7.5-kb VEGFR-1 mRNAs (21) are expressed constitutively by endothelial cells in culture (37, 38). The second band of 3.4 kb recognized by VEGFR-2 cDNA represents an alternative transcript, as previously reported (37). Furthermore, neuropilin-1 has been recently demonstrated to be a specific co-receptor of VEGFR-2 for the binding of VEGF-A_{165} isoform (27). TNF-α stimulation of endothelial cells from human umbilical cord for 24 h induced an increase in VEGFR-2 (Fig. 1) and neuropilin-1 (Fig. 3*B*) mRNA levels. The effect on VEGFR-2 transcript was observed in different conditions of culture: medium supplemented with 5% FCS (Fig. 1*B*), 20% FCS (Fig. 1*C*), or 1% FCS associated with 1% BSA (Fig. 1*A*). In medium 199 containing 20% FCS, TNF-α was active at 1 ng/ml. As shown in Fig. 2, the levels of VEGFR-2 mRNA began to increase after 4 h of incubation with TNF-α (20 ng/ml), reached a maximum level after around 24 h and was gradually reduced after 48 h. TNF-α was also active on foreskin microvascular endothelial cells. In the basal condition, these cells did not express amount of mRNA detectable by Northern technique. However, after treatment with TNF-α, the VEGFR-2 mRNA was evident in microvascular endothelial cells (Fig. 3*A*). The expression of VEGFR-1, the second receptor expressed on endothelial cell membrane, was not modified by TNF-α treatment (Fig. 3*B*).

Since it has been reported that TNF-α reduces the VEGFR-2 mRNA expression in human endothelial cells (39), two North-

[2] H. Gerber and J. Park, personal communication.

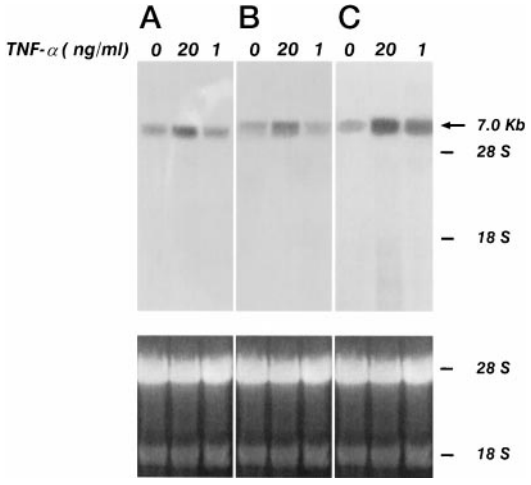

FIG. 1. **Effect of TNF-α on VEGFR-2 mRNA expression in human endothelial cells.** Northern blot analysis of total RNA extracted from confluent endothelial cells stimulated for 24 h with TNF-α in medium 199 containing 1% FCS and 1% BSA (*A*), 5% FCS (*B*), or 20% FCS (*C*). Fifteen μg of total RNA were run in a formaldehyde-agarose gel and, after blotting to Duralon membrane, hybridized to VEGFR-2 cDNA labeled with [α-^{32}P]dCTP. Transcripts have been visualized by autoradiography. The *lower panel* displays an image of the respective ethidium bromide-stained nylon membranes to demonstrate even loading and transfer. This experiment is representative of three performed with similar results.

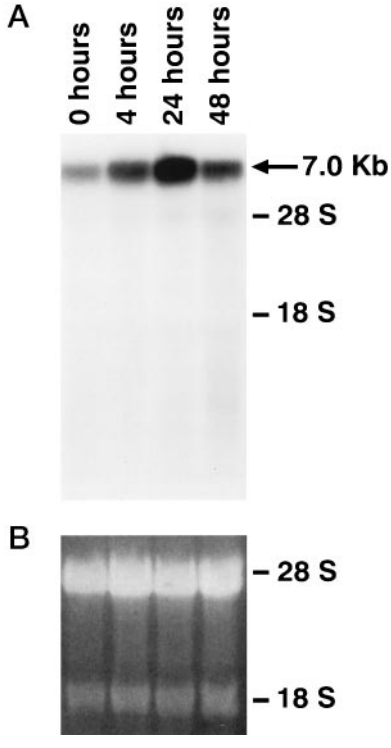

FIG. 2. **Time course of TNF-α-induced up-regulation of VEGFR-2 mRNA expression in human endothelial cells.** *A*, VEGFR-2 mRNA level from starved and confluent endothelial cells stimulated with 20 ng/ml TNF-α in medium 199 containing 20% FCS was determined by Northern blotting as detailed in the legend to Fig. 1. *B*, an image of the respective ethidium bromide-stained nylon membranes to demonstrate even loading and transfer. This experiment is representative of two experiments performed with similar results.

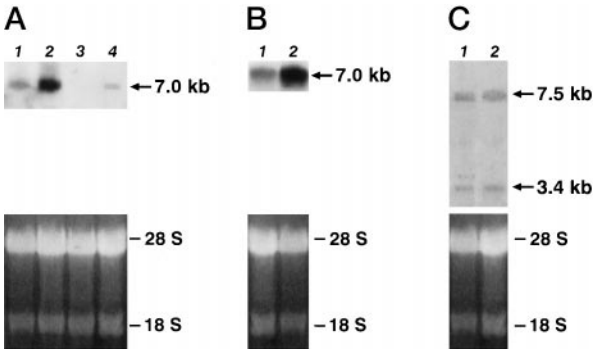

FIG. 3. **Effect of TNF-α on expression of VEGFR-2 mRNA in human foreskin endothelial cells (*A*), of neuropilin-1 mRNA (*B*), and of VEGFR-1 mRNA in human endothelial cells from umbilical cord (*C*).** *A*, confluent human endothelial cells from vein cord (*lanes 1* and *2*) and from foreskin endothelium (*lanes 3* and *4*) were stimulated for 24 h with 20 ng/ml TNF-α in medium 199 containing 10% (foreskin endothelium) or 20% FCS (umbilical endothelial cells) (*lanes 2* and *4*) or vehicle alone (*lanes 1* and *3*). VEGFR-2 mRNA was determined by Northern blotting as detailed in the legend to Fig. 1. *B*, confluent human endothelial cells from vein cord were stimulated for 24 h with 20 ng/ml TNF-α in medium 199 containing 5% FCS (*lane 2*) or vehicle alone (*lane 1*). Neuropilin-1 mRNA was determined by Northern blotting as detailed in the legend to Fig. 1. *C*, confluent human endothelial cells from vein cord were stimulated for 24 h with 20 ng/ml TNF-α in medium 199 containing 5% FCS (*lane 2*) or vehicle alone (*lane 1*). VEGFR-1 mRNA was determined by Northern blotting as detailed in the legend to Fig. 1. The *lower panels* display an image of the respective ethidium bromide-stained nylon membrane to demonstrate even loading and transfer. These experiments are representative of two performed with similar results.

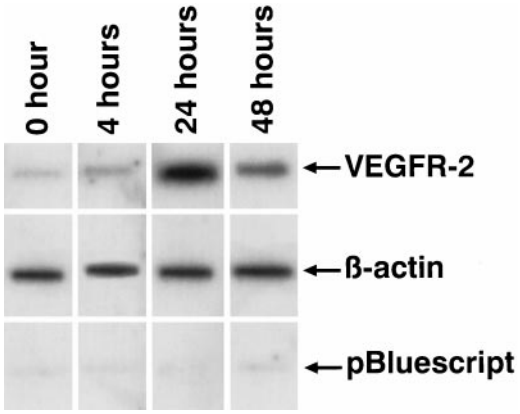

FIG. 4. **Nuclear run-on analysis after exposure of endothelial cells to TNF-α.** Nuclei were prepared from cells incubated in medium 199 containing 5% FCS with or without 20 ng/ml TNF-α. Transcription in the isolated nuclei was analyzed by hybridization of ^{32}P-labeled RNA to 15 μg of VEGFR-2, β-actin, and pBluescript cDNAs immobilized on nitrocellulose membrane. This experiment is representative of two experiments performed with similar results.

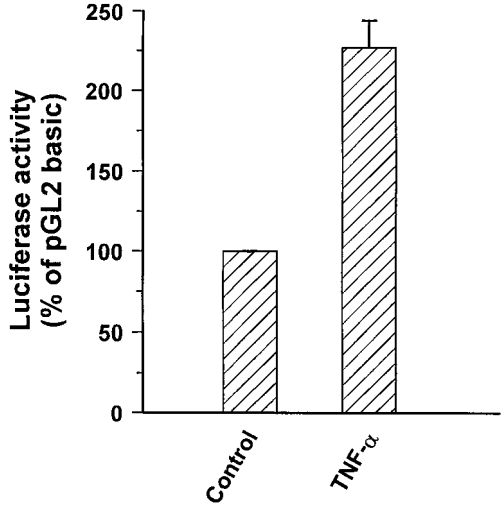

FIG. 5. **Activity of VEGFR-2 promoter-based luciferase construct in human fibrosarcoma 8387 cell line.** Cells were transfected with 3 μg of pGL2basicFlk or pGL2basic and subsequently treated for 4 h with 20 ng/ml TNF-α or vehicle alone. Extracts were analyzed for luciferase level. Transfection efficiency was corrected by cotransfection with pSVβgal. Mean ± S.D. of three experiments performed in duplicate is shown.

ern blots have been performed in two different laboratories (Dr. M. Introna, "Mario Negri" Institute, Milano, Italy; Dr. V. van Hinsbergh, TNO Prevention and Health, Leiden, The Netherlands) with results similar to those shown in Fig. 1. Furthermore, a marked increase of VEGFR-2 mRNA was also observed in porcine aortic endothelial cells transfected with the VEGFR-2 gene and stimulated with TNF-α (20 ng/ml) for 12 h (data not shown).

Induction of VEGFR-2 Gene Expression by TNF-α—In order to investigate whether TNF-α activates transcription of the VEGFR-2 gene in endothelial cells, nuclear run-on assay was performed. Nuclei were prepared from endothelial cells cultured with medium alone and with 20 ng/ml of TNF-α for 4, 24, and 48 h, and RNAs transcribed from these nuclei were hybridized with VEGFR-2 cDNA. We observed that TNF-α increased the transcription rate of VEGFR-2, without affecting the transcription of β-actin gene and of pBluescript plasmid, used as negative control (Fig. 4). The densitometric analysis done on three independent run-on assays showed that the transcriptional rate of the VEGFR-2 gene was respectively elevated of 1.2 ± 0.6-, 6.0 ± 0.5-, and 3.5 ± 0.3-fold after 4, 24, and 48 h of TNF-α incubation.

To further confirm that TNF-α regulates the transcription of VEGFR-2 gene, the mouse VEGFR-2 promoter-based reporter gene construct (36) was transiently transfected in 8378 human fibrosarcoma cells, which were treated for 4 h with TNF-α (20 ng/ml). This cell line was selected instead of endothelial cells, because it was more easily transfected with the construct than endothelial cells and expresses a functional active TNF receptor (29). Analysis of the respective luciferase expressions in untreated and TNF-α-stimulated cells revealed notable basal activity of the pGL2basicFlk-1 construct, which was increased after treatment with the cytokine by a factor of 2 (Fig. 5).

Human and mouse VEGFR-2 promoter contain five Sp1 elements (40), a transcription factor involved in TNF-α-induced gene expression in endothelial cells (41). We evaluated the role of Sp1 elements in the regulation of VEGFR-2 transcription by incubating endothelial cells with mithramycin, an inhibitor of the binding of the transcription factor to the CG box (7, 31). Fig. 6 shows that mithramycin at 1 and 10 nM inhibited the TNF-α-induced expression of VEGFR-2, suggesting that Sp1 binding sites in the promoter are involved in up-regulation of VEGFR-2 molecules.

TNF-α Increased the Expression of VEGFR-2 on Endothelial Cell Surface—The effect of TNF-α on the up-regulation of VEGFR-2 expression was further investigated with the analysis of ^{125}I-VEGF-A_{165}-specific binding at equilibrium on the endothelial cell surface. Since TNF-α does not up-regulate VEGFR-1 (Fig. 3*C*), the binding studies were performed by incubating endothelial cells with ^{125}I-VEGF-A_{165} concentrated to 50 pM or higher. This experimental condition excluded the analysis of the binding of VEGF-A_{165} to VEGFR-1 (42), which has a K_d ranging from 9 to 16 pM (24, 43), and indicated a single high affinity VEGF-A_{165} binding site on endothelial cells (Fig. 7*A*). Cell treatment for 24 h with TNF-α (20 ng/ml) in medium 199 containing 5% FCS produced a significant increase in ^{125}I-VEGF binding to cell surface. Nonlinear regression analysis

(Fig. 7*B*) of the data reported in Fig. 7*A* indicated a K_d = 137 ± 23 pM (n = 3) in untreated cells and a K_d = 122 ± 21 pM (n = 3) in TNF-α-treated cells. In contrast, TNF-α triplicates the number of binding sites expressed on cell membrane (in untreated cells, B_{max} = 79 ± 14 fmol; in TNF-α-treated cells, B_{max} = 243 ± 16 fmol, n = 3). Similar results have been obtained with TNF-α-treated endothelial cells in presence of 20% FCS (data not shown).

To analyze the expression of the protein encoded by VEGFR-2 gene, endothelial cells were treated with TNF-α for 24 h, and then the proteins from cell lysate were separated by SDS-polyacrylamide gel electrophoresis and probed with anti-VEGFR-2 antibodies. Fig. 8 shows that TNF-α treatment increased the amount of a 210-kDa protein recognized by an antibody anti-VEGFR-2. TNF-α did not modify the expression of proteins recognized by antibody anti-VEGFR-1 (data not shown).

Effect of TNF-α on VEGF-A_{165}-induced Endothelial Cell Migration—VEGF-A_{165} induced in a dose-dependent manner the migration of endothelial cells as evaluated by the Boyden chamber technique in agreement with previous observations (24, 44). The maximal migration was obtained with a concentration of VEGF-A_{165} of 10 ng/ml (3.7-fold the control value, $p < 0.05$). TNF-α alone did not influence endothelial cell migration. However, when endothelial cells were stimulated for 24 h with TNF-α (20 ng/ml) in the presence of 20% FCS (Fig. 9) or lower FCS amounts (1 and 5%; not shown), they showed an increased motility after challenge with VEGF-A_{165}. TNF-α treatment sensitized endothelial cells to an ineffective dose of VEGF-A_{165} (1 ng/ml) and was able to double the number of migrating cells stimulated with VEGF-A_{165} used at optimal concentration (10 ng/ml) (Fig. 9). The migration of endothelial cells triggered by hepatocyte growth factor (28) was not enhanced by cell treatment with TNF-α, suggesting that the effect of this cytokine is specific for VEGF-A_{165} (Table I).

Effect of TNF-α on VEGF-A_{165}-induced Wound Repair in Endothelial Cell Monolayers—A wound healing assay *in vitro*, *i.e.* the ability of filling artificial gaps created in cell monolayers, requires both cell growth and activation of cell movements. In preliminary experiments, we demonstrated that VEGF-A_{165} used at 10 ng/ml induced repair of mechanical wounds generated in human endothelial cell monolayers within 24 h but was ineffective at 1 ng/ml. The experiment given in Fig. 10 shows that pretreatment of the cells with TNF-α (20 ng/ml) for 24 h evidently enhances the VEGF-A_{165}-induced wound repair. Table II provides a quantitative analysis of cells migration into and across the wound, supporting the qualitative experiment in Fig. 10.

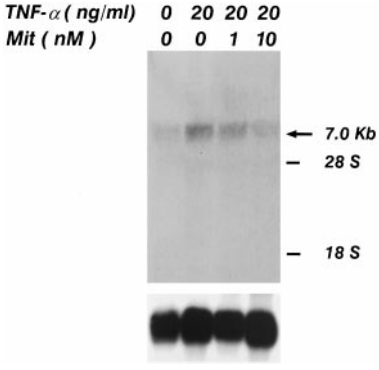

FIG. 6. **Effect of mithramycin on endothelial cell increase in VEGFR-2 mRNA levels by TNF-α.** Cells were stimulated in medium 199 supplemented with 5% FCS with 20 ng/ml TNF-α in the absence or presence of 1 and 10 nM mithramycin. The cellular levels of VEGFR-2 transcript were determined by Northern blotting as detailed in the legend to Fig. 1. The *lower panel* displays the Northern blot performed with β-actin cDNA. This experiment is representative of two experiments done with similar results.

DISCUSSION

TNF-α is an inflammatory cytokine with a wide spectrum of biological activities including angiogenesis (1, 45, 46). TNF-α acts particularly on the formation of new vessels by multiple indirect ways instead of promoting directly the sprout of endothelial cells and their growth, as the direct angiogenic inducers. The release of direct angiogenic molecules and up-regulation of proteolytic systems seem to be the biological events triggered by TNF-α to participate in angiogenesis (6, 7, 10, 12, 13, 17–19). In this report, we add a new piece to this mosaic, showing that TNF-α increases the transcription rate of the VEGFR-2 gene in vascular endothelial cells, resulting in augmented number of molecules expressed on cell surface and enhances the biological response of endothelial cells to VEGF-A_{165}. This statement is based on five major observations: 1) in the binding analysis performed with ^{125}I-VEGF-A_{165} concentrated to 50 pM or higher in order to render negligible the contribution of VEGFR-1 (42), TNF-α triples the number of high affinity binding sites for VEGF-A_{165} on endothelial membrane without affecting the affinity of the receptor for the ligand; 2) this effect is coupled to an early increase of mRNA expression of VEGFR-2, whereas the VEGFR-1 transcript is unchanged; 3) the up-regulation of VEGFR-2 mRNA results from an increase of the transcription as demonstrated by nuclear run-on assay and by the mouse VEGFR-2 promoter activation transfected in a human fibrosarcoma cell line responsive to and challenged with TNF-α; 4) human endothelial cells pretreated with TNF-α are more responsive to VEGF-A_{165} than untreated cells in terms of

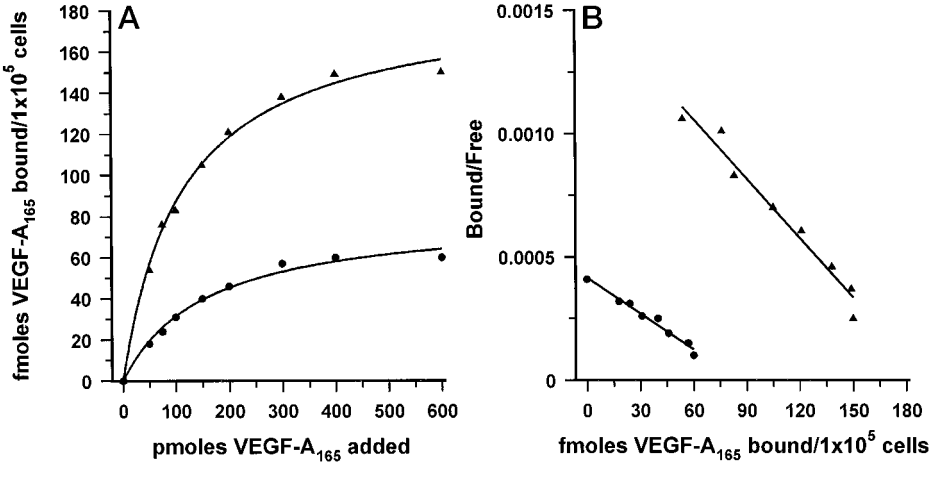

FIG. 7. **Effect of TNF-α on binding of radiolabeled VEGF-A_{165} to human endothelial cells.** *A*, specific ligand binding curve. Monolayers were incubated with TNF-α (20 ng/ml) (▲) or vehicle alone (●) for 24 h in medium 199 containing 5% FCS. After washes, cells were incubated with indicated concentrations of ^{125}I-VEGF-A_{165} for 2 h at 4 °C in the presence of a 100-fold excess of cold ligand. *B*, Scatchard plot of the data reported in *A*. The data shown are representative of three experiments.

migration and ability to repair a wounded monolayer; 5) the enhancement effect of TNF-α on endothelial cell migration is not observed when hepatocyte growth factor, an activator of endothelial motility (28), is used as stimulus, suggesting a relative specificity of the system.

The effect of TNF-α on mRNA expression of VEGFR-2 is dose-dependent and consistently detected with 1 ng/ml cytokine. The mRNA expression appears within 4 h after TNF-α stimulation and persists up to 24 h and then declines to basal level within 48 h. This time course is similar to that of other genes activated by TNF-α to direct endothelial cells toward a proinflammatory phenotype (47). Among these genes, E-selectin and vascular cell adhesion molecule-1 regulate leukocyte transmigration, but the soluble forms of the encoded proteins have been reported to be angiogenic too (48).

The control of VEGFR-2 transcription is entrusted by a promoter characterized by putative binding sites for AP-2, Sp1, and NF-κB transcription factors (36, 40). The TNF-α-induced activation of VEGFR-2 was inhibited by mithramycin, an inhibitor of Sp1 interaction with its consensus sequence (7, 31), suggesting that the rapid increase of VEGFR-2 might thus be mainly mediated through the activation of Sp1 in endothelial cells. Recently, it has been reported in endothelial cells that Sp1 is the major nuclear protein binding to VEGFR-2 promoter (49) and that TNF-α up-regulates Sp1 transcription and expression with a time course similar to that described in this study for VEGFR-2 (41). Sp1 oligonucleotide antisense inhibits the stimulating effect of TNF-α on VEGF-A production by endothelium and on *in vitro* angiogenesis (18). Since the VEGF-A promoter has Sp1 binding sites (50), Yoshida and co-workers have hypothesized that inhibition of VEGF-A synthesis may be the mechanism by which TNF-α-induced *in vitro* angiogenesis is affected (18). Viewed in light of the results shown here, it is also reasonable to explain the inhibition of Sp1 oligonucleotide antisense on angiogenesis as an effect on the mechanisms leading to the up-regulation of VEGFR-2 induced by TNF-α.

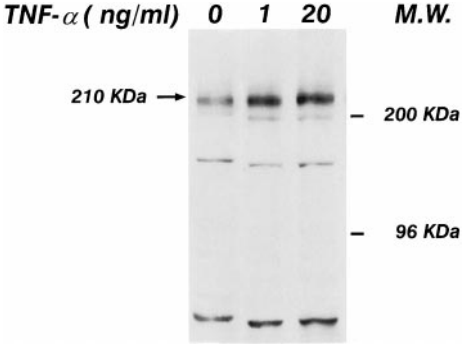

FIG. 8. **Western blot analysis of VEGFR-2 in human endothelial cells stimulated with TNF-α.** Confluent endothelial cell monolayers were treated for 24 h with TNF-α in medium 199 containing 5% FCS. 200 μg of lysed proteins were separated by SDS-polyacrylamide gel electrophoresis (7%), blotted onto polyvinylidene difluoride membrane, probed with rabbit anti-VEGFR-2 antibody, and detected by enhanced chemiluminescence.

TNF-α also increases the transcription of *neuropilin-1*, which enhances the binding of VEGF-A_{165} to VEGFR-2 and VEGF-A_{165}-mediated chemotaxis (27). The binding of VEGF-A_{165} to neuropilin-1 is mediated through the amino acid sequence encoded by exon-7, absent in other VEGF-A isoforms (25), suggesting the high specificity of this co-receptor for VEGF-A_{165} (27). Further experiments could discriminate whether the effect of TNF-α is restricted to VEGF-A_{165} or also present in other isoforms.

The observed up-regulation of VEGFR-2 by TNF-α is in disagreement with the results published by Patterson and co-workers (39), who demonstrated that TNF-α down-regulates VEGFR-2 expression in human endothelial cells from veins or arteries. This discrepancy could be due to differences in experimental conditions. However, human microvascular endothelial cells from omental tissue increase VEGFR-2 mRNA when challenged with TNF-α (18).

Therefore, this study brings new insight into the conditions regulating the endothelial cell response to VEGF-A_{165}. Previous studies on the mechanism responsible for the regulated expression of VEGFR-2 have focused on TGF-β1 (51) or hypoxia (42). Notably, neutralizing anti-TNF-α antibodies did not neutralize the up-regulation of VEGFR-2 by conditioned media from hypoxic cells (42). Hypoxia does not affect directly the VEGFR-2 promoter (36), but the up-regulation is mediated by an unknown factor present in ischemic tissues (42). TGF-β1 decreases the expression of VEGFR-2 after a prolonged time of incubation, by a presently unknown molecular mechanism (51). Indeed, TNF-α is the first identified cytokine that increases the endothelial cell response to VEGF-A_{165} by a direct effect on the VEGFR-2 transcription.

Our *in vivo* preliminary experiments agree with our *in vitro*

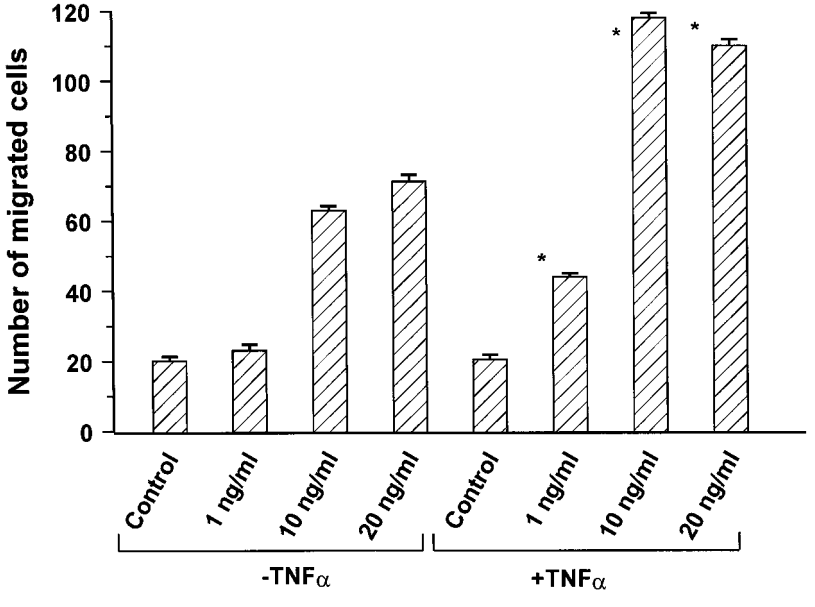

FIG. 9. **Effect of TNF-α on migration of human endothelial cells elicited by VEGF-A_{165}.** The migration of cells was measured by the modified Boyden chamber technique, as described under "Experimental Procedures." Human endothelial cells were treated with 20 ng/ml TNF or vehicle alone for 24 h in medium 199 with 5% FCS. Suspended cells (1.25×10^5) in medium 199 containing 1% FCS were seeded in the upper compartment of the chamber, and VEGF-A_{165} suspended in the same medium containing 1% FCS was placed in the lower compartment. Cells that migrated after 6 h of incubation to the lower surface of the filter were counted after coding samples. The *numbers* are the mean ± S.D. of eight experiments performed in triplicate. *, $p > 0.05$.

TABLE I
Effect of TNF-α on endothelial cell migration stimulated by VEGF-A_{165}, and hepatocyte growth factor

Endothelial cells were pretreated with TNF-α (20 ng/ml) or vehicle alone for 24 h and then stimulated in Boyden's chamber with 10 ng/ml each molecule in medium 199 containing 1% FCS and processed as detailed in the legend of Fig. 1. The values are the means ± S.D. of three experiments done in triplicate.

	Number of migrated cells	
	Without TNF-α	With TNF-α
None	20.1 ± 1.2	20.4 ± 1.3
VEGF-A_{165}	79.1 ± 1.8	117.3 ± 1.6[a]
Hepatocyte growth factor	71.8 ± 1.5	73.1 ± 2.2

[a] $p < 0.05$.

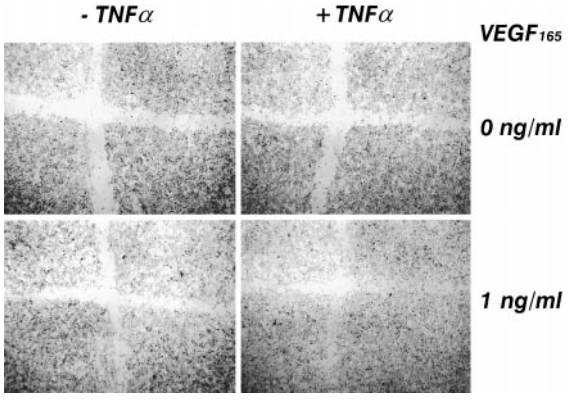

FIG. 10. **Effect of TNF-α on wound healing of human endothelial cell monolayer induced by VEGF-A_{165}.** Confluent endothelial cell monolayers were treated for 24 h with TNF-α (20 ng/ml) in medium 199 containing 5% FCS and washed twice with medium without FCS. The monolayer was wounded with a cross-shaped scratch and stimulated with VEGF-A_{165} (1 ng/ml) for 24 h in medium 199 containing 3% BSA. At the end of incubation, cells were fixed and stained by crystal violet. Magnification was ×2. The *picture* is representative of five experiments with similar results.

TABLE II
Effect of TNF-α on endothelial cell wound repair stimulated by VEGF-A_{165}

Endothelial cell monolayer was treated with TNF-α (10 ng/ml) or vehicle alone for 24 h and then wounded with a razor blade. After washes, cells were stimulated with VEGF-A_{165} (1 ng/ml) in medium 199 containing 3% BSA and processed as described in the legend of Fig. 2. At the end of stimulation, the wounded area was recorded with a still video camera recorder. Cells in 10 fields of 1 mm^2 were counted with an image analyzer. The values are the means ± S.D. of 10 fields in one experiment representative of three performed with similar results.

	Number of cells/mm^2	
	Without TNF-α	With TNF-α
None	1103 ± 350	1204 ± 267
VEGF-A_{165}	1679 ± 403	4093 ± 167[a]

[a] $p < 0.05$.

results. The treatment of DBA2 mice with TNF-α (750 ng intraperitoneally followed by a second dose 3 days after) allows an ineffective angiogenic concentration of VEGF-A_{165} to promote vascularization in a Matrigel plug (52) injected subcutaneously on the day of the second TNF-α injection (vehicle-treated mice were injected with 5 ng of VEGF-A_{165}/0.75 ml of Matrigel ($n = 5$), and vascularized area was 6 ± 4% of total Matrigel area; vehicle-treated mice were injected with 0.75 ml of Matrigel ($n = 3$), and vascularized area was 3 ± 2% of total Matrigel area; TNF-α-treated mice were injected with 5 ng of VEGF-A_{165}/0.75 ml of Matrigel ($n = 5$), and vascularized area was 34 ± 10% of total Matrigel area; TNF-α-treated mice were injected with 0.75 ml of Matrigel ($n = 5$), and vascularized area was 7 ± 3% of total Matrigel area). Involvement of VEGFR-2/VEGF-A system has recently been demonstrated *in vivo* in cancer disease and in chronic inflammation (44, 53, 54), processes in which TNF-α is markedly up-regulated (55–57). Furthermore, we have recently demonstrated that VEGFR-2 is also the receptor of Tat (58), a protein of immunodeficiency virus-1 involved in the angiogenesis associated with Kaposi's sarcoma (59). Since our results demonstrate that TNF-α presents as a potent inducer of VEGFR-2 synthesis, the TNF-α-mediated up-regulation of the unique VEGF receptor capable to mediate mitogenic and motogenic signals inside endothelial cells (22, 24) is likely to play an important role in the initiation and maintenance of angiogenesis and increased vascular permeability in these conditions.

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Tumor Necrosis Factor-α Regulates Expression of Vascular Endothelial Growth Factor Receptor-2 and of Its Co-receptor Neuropilin-1 in Human Vascular Endothelial Cells

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