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 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 125I-VEGF-A165 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-A165. 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) 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-α (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-
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 KDR/Flk-1 (21) and VEGFR-2 by PTK7 (22, 23), but recent findings suggest that the latter receptor, VEGFR-2, is more involved in angiogenesis and is the receptor for the mitogenic and chemotactic effect of VEGF-A165 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-A165. An augmented expression of VEGF-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 grown 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 hepatin (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 (1–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 VEGF-2 (24) were cultured in Ham’s F-12 (Sigma) supplemented with 10% FCS. Human foreskin microvascular endothelial cells transfected with human VEGFR-2 (24) were cultured in Ham’s F-12 (Sigma) supplemented with 10% FCS. Fibroblasts (29) from 300 μl at 30 °C for 30 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 Tris, pH 7.4, 10 mM EDTA, 0.2% SDS) and SDS (5% final concentration), incubation was continued at 37 °C for 30 min. Extracted RNA was resuspended in TES buffer (10 mM Tris, pH 7.4, 10 mM EDTA, 0.2% SDS) at 4 °C for 30 min. Extracted RNA was resuspended in TES buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.1 mM EDTA) and frozen in liquid N₂. In situ transfection and isolation of the resulting nuclear RNA were performed as described by Ikeda et al. (35). Two-hundred μl of frozen nuclear were thawed and mixed with 200 μl of 2× reaction buffer (10 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM MgCl₂, 0.1 mM EDTA) and frozen in liquid N₂. The puzzling effects of TNF-α on the expression of VEGF receptors and on the biological activities elicited by VEGF-A165, the following experimental conditions have been used: confluent endothelial cell growth at a CO₂ level of 5% in atmosphere air was treated with 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 200 μM Heps containing 10% human serum, 10% newborn calf serum, endothelial cell growth supplement (150 μg/ml), and porcine hepatin (5 μg/ml).

Experimental Design—To verify the effects of TNF-α on the expression of VEGF receptors and on the biological activities elicited by VEGF-A165, the following experimental conditions have been used: confluent endothelial cell growth at a CO₂ level of 5% in atmosphere air was treated with 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 200 μM Heps containing 10% human serum, 10% newborn calf serum, endothelial cell growth supplement (150 μg/ml), and porcine hepatin (5 μg/ml). Alternatively, cells were stimulated with VEGF-A165 (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 thiocyanate extraction and centrifugation and denatured through cesium chloride (32). Equal amounts of total RNA (15 μg/lane) were electrophoresed in 1% agarose gels containing 6.3% formamide 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 H₂O light (0.5 J/cm²) and prehybridized for 4 h at 42 °C in 50% formamide (Sigma), 200 mM NaCl, 150 mM sodium citrate, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40) and allowed to stand on ice for 5 min. Then the filters were washed at 500 × g for 5 min at 4 °C. Subsequent steps were performed at 4 °C. The following hybridization and washing conditions were used: 100 μg/ml of labeled cDNA (10 μg/ml Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40) and allowed to stand on ice for 5 min. Then the filters were washed at 500 × g for 5 min 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 MgCl₂, 0.1 mM EDTA) and frozen in liquid N₂. In situ transfection and isolation of the resulting nuclear RNA were performed as described by Ikeda et al. (35). Two-hundred μl of frozen nuclear were thawed and mixed with 200 μl of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 300 μM KCl 10 mM dithiothreitol, 400 units/ml placental ribonuclease inhibitor (Strategene), 20 μM creatine phosphate (Sigma), 200 μg/ml creatine phosphokinase (Sigma), 1 mM a mixture each of ATP, CTP, and GTP (Strategene), and 100 μl of [α-33P]UTP (3000 Ci/mmól, Amersham). Samples were run on 300 μl at 30 °C for 30 min in the presence of 20 units of DNase I (RNAse-free, Life Technologies, Inc.). Filtered 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 VEGF-2 mRNA was standardized by comparison with the amount of β-actin mRNA. Nuclei run-on—Nuclei were isolated from cultured endothelial cells essentially according to Ref. 34. Briefly, cells (2 × 10⁷ 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 × g for 5 min at 4 °C. Subsequent steps were performed at 4 °C. The nuclei were resuspended in 200 μl of glycerol storage buffer (10 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM MgCl₂, 0.1 mM EDTA) and frozen in liquid N₂.

Binding Assay and Analysis—Recombinant VEGF-A₁₆₅ (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-A₁₆₅ was iodinated (5 min, 4 °C) with 1 μCi of [¹²⁵I] (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 Sephacryl-100. The specific activity of the tracer was 90,000 cpm/μg. ¹²⁵I-VEGF-A₁₆₅ 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 μM well of binding medium (medium 199 containing 20 mM Hepes buffer, pH 7.4, 0.1% BSA, 100 μg/ml soybean trypsin inhibitor, 1 M NaCl, 0.32 M NaCl, 3 mM MgCl₂, 0.1 mM EDTA) and then with 100 μg/ml anti-VEGFR-2 antibody (C-1155, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and detected by ECL (Amersham).
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-A165 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. 3A) mRNA levels. The effect on VEGFR-2 transcript was observed in different conditions of culture: medium supplemented with 5% FCS (Fig. 1A) or vehicle alone in medium 199 containing 5% FCS. After washes, the monolayer was wounded with a razor blade (lesion surface: 20 mm²) as described (28) and incubated in medium 199 containing 3% BSA with or without VEGF-A165. 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 mm² randomly selected, with a Cosmoczone image analyzer (Nikon, Tokyo, Japan).

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 VEGF-2 cDNA labeled with [32P]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.
of Sp1 elements in the regulation of VEGFR-2 transcription by TNF-α 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. 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). VEGFR-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. C. 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.

**Induction of VEGF-2 Gene Expression by TNF-α**—In order to investigate whether TNF-α activates transcription of the VEGF-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 VEGF-2 cDNA. We observed that TNF-α increased the transcription rate of VEGF-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 VEGF-2 gene was respectively elevated of 6.0-, 10.0-, and 16.0-fold after 4, 24, and 48 h of TNF-α incubation.

To further confirm that TNF-α regulates the transcription of VEGF-2 gene, the mouse VEGF-2 promoter-based reporter gene construct (36) was transiently transfected in 8387 human fibrosarcoma 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.

**TNF-α Increased the Expression of VEGFR-2 on Endothelial Cell Surface**—The effect of TNF-α on the up-regulation of VEGF-2 expression was further investigated with the analysis of 125I-VEGF-A165-specific binding at equilibrium on the endothelial cell surface. Since TNF-α does not up-regulate VEGF-1 (Fig. 3C), the binding studies were performed by incubating endothelial cells with 125I-VEGF-A165 concentrated to 50 pM or higher. This experimental condition excluded the analysis of the binding of VEGF-A165 to VEGF-1 (42), which has a Kd ranging from 9 to 16 pM (24, 43), and indicated a single high affinity VEGF-A165 binding site on endothelial cells (Fig. 7A). Cell treatment for 24 h with TNF-α (20 ng/ml) in medium 199 containing 5% FCS produced a significant increase in 125I-VEGF binding to cell surface. Nonlinear regression analysis...
TNF-α Up-regulates VEGFR-2 Expression and Function

(22132) in TNF-α-treated cells. In contrast, TNF-α triplicates the number of binding sites expressed on cell membrane (in untreated cells, \( B_{\text{max}} = 79 \pm 14 \text{ fmol} \); in TNF-α-treated cells, \( B_{\text{max}} = 243 \pm 16 \text{ fmol} \)). 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-A165-induced Endothelial Cell Migration—VEGF-A165 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-A165 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-A165. TNF-α treatment sensitized endothelial cells to an ineffective dose of VEGF-A165 (1 ng/ml) and was able to double the number of migrating cells stimulated with VEGF-A165 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-A165 (Table I).

Effect of TNF-α on VEGF-A165-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-A165 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-A165-induced wound repair. Table II provides a quantitative analysis of cells migration into and across the wound, supporting the qualitative experiment in Fig. 10.

**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 VEGF-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-A165. This statement is based on five major observations: 1) in the binding analysis performed with 125I-VEGF-A165 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-A165 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-A165 than untreated cells in terms of

**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 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.

**FIG. 7.** Effect of TNF-α on binding of radiolabeled VEGF-A165 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 125I-VEGF-A165 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.
m migrants 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 promoter is 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.

TNF-α also increases the transcription of neuropilin-1, which enhances the binding of VEGF-A165 to VEGFR-2 and VEGF-A165-mediated chemotaxis (27). The binding of VEGF-A165 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-A165 (27). Further experiments could discriminate whether the effect of TNF-α is restricted to VEGF-A165 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-A165. Previous studies on the mechanism responsible for the regulated expression of VEGF-A2 have focused on TGF-β1 (51) or hypoxia (42). Notably, neutralizing anti-TNF-α antibodies did not neutralize the up-regulation of VEGF-A 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 VEGF-A 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-A165 by a direct effect on the VEGF-2 transcription.

Our in vivo preliminary experiments agree with our in vitro
TABLE I

Effect of TNF-α on endothelial cell migration stimulated by VEGF-A165 and hepatocyte growth factor

| Condition               | Without TNF-α | With TNF-α |
|-------------------------|---------------|------------|
| None                    | 20.1 ± 1.2    | 20.4 ± 1.3 |
| VEGF-A165               | 79.1 ± 1.8    | 117.3 ± 1.6* |
| Hepatocyte growth factor| 71.8 ± 1.5    | 73.1 ± 2.2 |

* 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-A165 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-A165/0.75 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 = 5), and vascularized area was 3 ± 2% of total Matrigel area; TNF-α-treated mice were injected with 5 ng of VEGF-A165/0.75 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 VEGF-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 VEGF-R2 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 VEGF-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.

Acknowledgments—We thank Dr. Lena Claesson-Welsh (Uppsala University, Uppsala, Sweden), Dr. Martino Introna (Istituto “Mario Negri,” Milano, Italy), and Dr. P. M. Comoglio (Institute for Cancer Research, Torino, Italy), who provided porcine aortic endothelial cells transfected with VEGF-2, human fibrosarcoma 8378 cells, and human recombinant hepatocyte growth factor, respectively. Dr. Martino Introna and Victor van Hinsbergh provided mRNA of TNF-α-stimulated and control endothelial cells. VEGF-R2 and VEGF-R1 were kindly provided by Dr. Bruce Terman (Wyeth Ayerst Research, Pearl River, NY), and recombinant VEGF-A165 was provided by Dr. Herbert Weich (GBF, Braunschweig, Germany).

REFERENCES

1. Bussolino, F., Mantovani, A., and Persico, G. (1997) Trends Biochem. Sci. 22, 251–256
2. Folkman, J. (1995) Nat. Med. 1, 27–31
3. Risau, W. (1997) Nature 386, 671–674
4. Leibovich, S. J., Polverini, P. J., Shepard, H. M., Wiseman, D. M., Shively, V., and Norden, R. (1987) Nature 329, 630–632
5. Frater-Schroder, M., Risau, W., Hallmann, P., Gatschke, P., and Bohlen, P. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5277–5281
6. Montrucheu, G., Lupia, E., Battaglia, E., Passerini, G., Bussolino, F., Emanuelli, G., and Camussi, G. (1994) J. Exp. Med. 180, 377–382
7. Bryte, M., Ono, M., Izumi, H., Yasuda, S., Weiss, H. A., Kohno, K., and Kuwano, M. (1996) J. Biol. Chem. 271, 28220–28228
8. Defilippi, P., Truffa, G., Stefanuto, G., Altura, F., Silengo, L., and Tarone, G. (1991) J. Biol. Chem. 266, 7638–7645
9. Holzmann, L. B., Marks, R., and Dixit, V. M. (1990) Mol. Cell. Biol. 10, 5830–5838
10. Okamura, K., Sato, Y., Matsuda, T., Hamaanaka, R., Ono, M., Kohno, K., and Kuwano, M. (1991) J. Biol. Chem. 266, 19162–19165
11. Bussolino, F., Camussi, G., and Baglioni, C. (1988) J. Biol. Chem. 263, 11856–11861
12. Pandey, A., Shao, H., Marks, R. M., Polverini, P. J., and Dixit, V. M. (1995) Science 268, 567–569
13. Nakwath, P., and Stern, D. (1987) J. Exp. Med. 167, 740–749
14. Carmeliet, P., Ferreira, V. A., Pellegrini, S., Polleux, F., Kieckens, L., Gertenstein, M., Fahrig, M., Vandenhoek, A., Harpal, K., Eberhardt, C., Declerq, C., Pawling, J., Monls, L., Collen, D., Risau, W., and Nagy, A. (1996) Nature 380, 435–439
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*J. Biol. Chem.* 1998, 273:22128-22135.
doi: 10.1074/jbc.273.34.22128

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