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Involvement of Interleukin-8, Vascular Endothelial Growth Factor, and Basic Fibroblast Growth Factor in Tumor Necrosis Factor Alpha-Dependent Angiogenesis

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Tumor necrosis factor alpha (TNF-α) is a macrophage/monocyte-derived polypeptide which modulates the expression of various genes in vascular endothelial cells and induces angiogenesis. However, the underlying mechanism by which TNF-α mediates angiogenesis is not completely understood. In this study, we assessed whether TNF-α-induced angiogenesis is mediated through TNF-α itself or indirectly through other TNF-α-induced angiogenesis-promoting factors. Cellular mRNA levels of interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and their receptors were increased after the treatment of human microvascular endothelial cells with TNF-α (100 U/ml). TNF-α-dependent tubular morphogenesis in vascular endothelial cells was inhibited by the administration of anti-IL-8, anti-VEGF, and anti-bFGF antibodies, and coadministration of all three antibodies almost completely abrogated tubular formation. Moreover, treatment with Sp1, NF-κB, and c-Jun antisense oligonucleotides inhibited TNF-α-dependent tubular morphogenesis by microvascular endothelial cells. Administration of a NF-κB antisense oligonucleotide almost completely inhibited TNF-α-dependent IL-8 production and partially abrogated TNF-α-dependent VEGF production, and an Sp1 antisense sequence partially inhibited TNF-α-dependent production of VEGF. A c-Jun antisense oligonucleotide significantly inhibited TNF-α-dependent bFGF production but did not affect the production of IL-8 and VEGF. Administration of an anti-IL-8 or anti-VEGF antibody also blocked TNF-α-induced neovascularization in the rabbit cornea in vivo. Thus, angiogenesis by TNF-α appears to be modulated through various angiogenic factors, both in vitro and in vivo, and this pathway is controlled through paracrine and/or autocrine mechanisms.

Normal angiogenesis, which is self-limiting and strictly regulated, is often observed during wound healing as well as during the formation of the endometrium, corpus luteum, and placenta. Angiogenesis is also involved in various pathologic inflammatory and angiogenic diseases, including psoriasis, rheumatoid arthritis, diabetic retinopathy, arteriosclerosis, and solid tumors (13, 14). The development of new blood vessels depends upon a balance of angiogenic factors and angiogenic inhibitors (14, 18). Angiogenic factors include vascular endothelial growth factor (VEGF), acidic fibroblast growth factor, basic fibroblast growth factor (bFGF), angiogenin, placenta growth factor, epidermal growth factor (EGF)/transforming growth factor α (TGF-α), hepatocyte growth factor, and plasminogen activators; angiogenic inhibitors include alpha and beta interferons, interleukin-12 (IL-12), TGF-β, tissue inhibitor of metalloproteinases, platelet factor-4, prolactin fragment, and thrombospondin (12, 14, 18). In contrast, tumor necrosis factor alpha (TNF-α), a macrophage/monocyte-derived pluripotent mediator, can function as an angiogenic factor in one system and as an antiangiogenic factor in another system. TNF-α stimulates angiogenesis in the cornea and chorioallantoic membrane in vivo (15, 29). However, TNF-α blocks both basal and bFGF-stimulated growth in bovine aortic endothelial cells, bovine capillary endothelial cells, and human umbilical endothelial cells in culture (58, 62) and is also antiangiogenic in the cornea (3). Fajardo et al. (11) demonstrated that this difference may be due to TNF-α concentrations; a low dose of TNF-α induced angiogenesis, but a high dose inhibited it. Moreover, long exposures of human microvascular endothelial cells to TNF-α inhibited tubular morphogenesis in an in vitro model system with a concurrent morphologic change from a cobblestone-like to fibroblast-like appearance (25, 36) and increased expression of plasminogen activator inhibitor-1 (37). In contrast, shorter exposures to TNF-α induced tubular morphogenesis by human microvascular endothelial cells in this angiogenesis model system (64).

TNF-α has been reported to induce the expression of many immunologically relevant genes (27), possibly through two different TNF-α receptors, TNF-αR1 and TNF-αR2. In vascular endothelial cells, TNF-α increased the expression of endothelial surface antigens, tissue factor, IL-1, platelet activating factor, and HLA-A or -B (4, 32, 43, 53) but decreased the expression of proteoglycan (55). This TNF-α-dependent induction is thought to be mediated, in part, through activation of the nuclear transcription factor NF-κB (9, 34, 48, 61).

In human microvascular endothelial cells, we have found that TNF-α enhances the expression of bFGF, IL-6, and the low-density lipoprotein receptor gene (17) and activates AP-1 as well as NF-κB (23). Sp1 also appears to be involved in TNF-α-induced activation of the low-density lipoprotein receptor gene (17, 46). Ryuto et al. (56) reported that the TNF-α-dependent induction of a potent angiogenic factor, VEGF, is mediated through activation of Sp1 in human glioma cells. The

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diverse effects of TNF-α on angiogenesis may be due to either time-dependent or dose-dependent expression of positive and negative angiogenic factors. The paracrine and/or autocrine control of TNF-α-dependent angiogenesis in vitro as well as in vivo are discussed.

MATERIALS AND METHODS

Materials. IL-8, human IL-8 cDNA, and anti-IL-8 antibody (64), as well as human VEGF cDNA, EGF/TGF-α cDNA, human EGF receptor cDNA, and bFGF cDNA, have been previously described (56, 64). The anti-VEGF and anti-bFGF antibodies employed in the in vitro models for angiogenesis were purchased from R & D Systems (Minneapolis, Minn.); anti-EGF/TGF-α antibody was purchased from Oncogene Science Inc.; TNF-α was purchased from Mochida Pharmaceutical Co. (Tokyo, Japan); anti-Sp1 and anti-c-Jun antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). For the in vivo corneal assay, a rabbit anti-human VEGF polyclonal antibody was used (26). Polyclonal anti-p65 antiserum was generated by multiple immunizations of a New Zealand White rabbit with synthesized peptides and affinity purified as described previously (19, 20). [γ-^{32}P]dATP, [α-^{32}P]UTP, and [α-^{32}P]dCTP were obtained from Amersham. Ethylene vinyl acetate (EVA) was purchased from Takeda Chemical (Osaka, Japan).

Antisense oligonucleotides. Antisense and sense phosphorothioate analogs of the oligonucleotides to the 5′ ends of Sp1, NF-κB, and c-Jun, including the ATG initiation codon, were synthesized as previously described (35). Their sequences are as follows: Sp1 sense, 5′-CCATGGATGAAATGACAGCTGTGGTG-3′; Sp1 antisense, 5′-CACCACAGCTGTCATTTCATCCATGG-3′; NF-κB sense, 5′-ACCCCGGCCATGGACGAACTGTTCCCCCTC-3′; NF-κB antisense, 5′-GAGGGGGAACAGTTCGTCCCATGGCCGGGGT-3′; c-Jun sense, 5′-ACGTGAAGTGACGGACTGTTCTATGACTGC-3′; and c-Jun antisense, 5′-GCAGTCATAGAACAGTCCGTCACTTCACGT-3′.

Quantitative analysis of tube formation in human microvascular endothelial cells on a type I collagen gel. Human microvascular endothelial cells were isolated from surgically removed omental tissue samples as previously described (36, 45, 47). Cells were plated onto the surface of a type I collagen gel in M-199 medium containing 10% fetal bovine serum (FBS). When cells reached confluence, the medium was replaced with M-199 medium containing 1% FBS with or without factors and incubated for an additional 72 h. On the third day, phase-contrast microscopic pictures (magnification, ×200) were recorded with a still video camera recorder (R5000H; Fuji Photo Film Co., Tokyo, Japan), and the total length of tube-like structures per field was measured with a Cosmozone image analyzer (Nikon, Tokyo, Japan) as previously described (45, 47, 59). Eight random fields per dish were measured, and the total length per field was calculated.

Northern blot analysis. Human microvascular endothelial cells were grown in M-199 medium containing 10% FBS, followed by incubation in serum-free M-199 medium for 24 h. Thereafter, factors were added for the indicated periods. Harvested cells were suspended in 4 M guanidinium isothiocyanate–25 mM sodium citrate (pH 7.0)–0.5% Sarkosyl–0.1 M β-mercaptoethanol. Total RNA was extracted as previously described (1, 23, 56). RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde, transferred onto a nylon membrane (Hybond N+; Amersham), and UV cross-linked at 0.25 J/cm^2 with FLUO-LINK (Viler Lourmat, Marne-La-Vallee, France). The membrane was hybridized to ^{32}P-labeled DNA probes in Hybrisol (Oncor, Inc., Gaithersburg, Md.) at 42°C for 24 h and washed twice at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS), once in 1× SSC–0.1% SDS, and finally in 0.2× SSC–0.1% SDS. mRNA levels were quantified by densitometry with a Fujix BAS 2000 bioimage analyzer (Fuji Photo Film Co.).

Detection of mRNA by quantitative RT-PCR. Synthetic oligonucleotides were prepared on the basis of the published cDNA sequences of IL-8 receptor (IL-8R) (21, 42), fms-like tyrosine kinase-1 (flt-1) (7), fetal liver kinase-1 (flk-1) (38, 66), fms-like gene (flg; bFGF receptor 1) (8), and β2-microglobulin (41). The primer sequences of the oligonucleotides used for PCR were as follows: IL-8R 442, 5′-CATCAGTGTGTGGACCGTTACC-3′; 807, 5′-AGGAAGATGAGGACGACAGC-3′; flt-1 1069, 5′-AAGAGAGCTTCCGTAAGGCG-3′; 1448, 5′-GCATCCTCTTCAGTTACGTCC-3′; flk-I 665, 5′-ATGATGTGGTTCTGAGTCCG-3′; 1010, 5′-AGAGATTCCATGCCACTTCC-3′; flg 1139, 5′-ATCACTCTGCATGGTTCACC-3′; and 1375, 5′-ACACTGTTACCTGTCTGCGC-3′. First-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). RNAs (1 μg) and random hexamer (100 ng) were denatured at 65°C for 10 min and added to the reverse transcription (RT) mixture as instructed by the manufacturer. After incubation at 37°C for 1 h, cDNA from the RT mixture was subjected to PCR in a 10-μl volume containing 5 pmol each of the primer pair, 0.5 U of Ampli *Taq* Gold (Perkin-Elmer Co., Norwalk, Conn.), and 1 μCi of [α-^{32}P]dCTP with a DNA thermal cycler (Perkin-Elmer Co.). DNAs were denatured for 9 min at 95°C, followed by 30 PCR cycles. Each cycle included 30-s denaturation at 94°C, 30-s primer annealing at 55°C, and 45-s polymerization at 72°C. An 8-μl aliquot of each RT-PCR mixture was analyzed by electrophoresis on a 5% acrylamide gel. The gel was dried and exposed overnight at −80°C for autoradiography, and the radioactivities of mRNA levels were measured with a Fujix BAS 2000 bioimage analyzer.

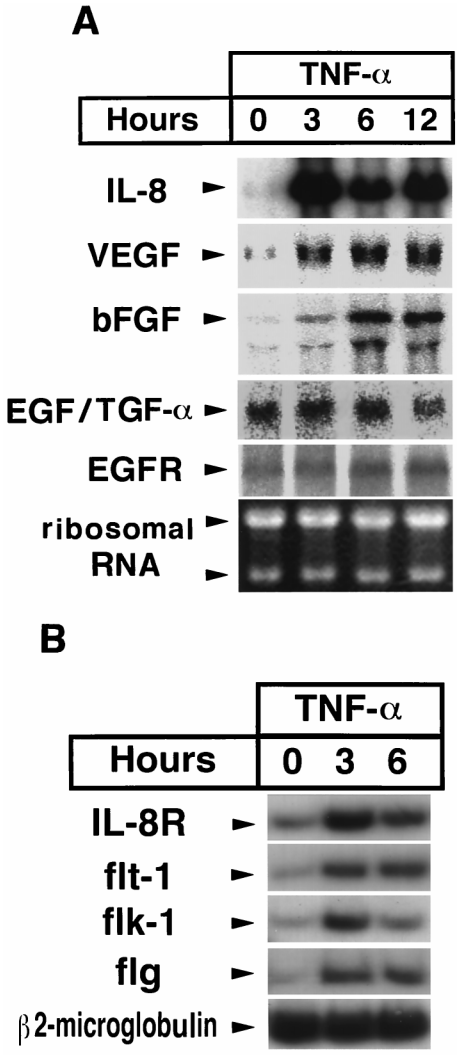


FIG. 1. Effects of TNF-α on the expression of IL-8, VEGF, bFGF, EGF/TGF-α, and their receptors. (A) Effects of TNF-α on the expression of IL-8, VEGF, bFGF, EGF/TGF-α, and EGF receptor (EGFR) mRNAs. Endothelial cells were incubated in serum-free M-199 medium for 24 h, exposed to TNF-α (100 U/ml) for 15 min, and then incubated in TNF-α-free medium for 2 h 45 min (3 h), 5 h 45 min (6 h), and 11 h 45 min (12 h). At the indicated times, cells were collected and mRNAs were hybridized with ^{32}P-labeled IL-8, VEGF, bFGF, EGF/TGF-α, and EGF receptor cDNA probes. The radioactivities of the corresponding areas were measured with a BAS 2000 bioimage analyzer. The cellular levels of IL-8, VEGF, bFGF, EGF/TGF-α, and EGF receptor mRNA were normalized to that of rRNA. (B) Effects of TNF-α on the expression of IL-8R, flt-1, flk-1, and flg mRNAs. At 3 and 6 h after TNF-α stimulation, cells were collected and 1 μg of RNA was reverse transcribed. Templates (100 [IL-8 and flt-1] and 5 [flk-1, flg, and β2-microglobulin] ng) were amplified and analyzed by autoradiography. The cellular levels of various receptor mRNAs were normalized to β2-microglobulin levels.

Western blot analysis. Protein fractions were electrophoresed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and blotted onto nitrocellulose filters. The nitrocellulose filters were developed by chemiluminescence according to the enhanced chemiluminescence protocol (Amersham) (64).

ELISA of IL-8, VEGF, and bFGF. The concentrations of IL-8, VEGF, and bFGF in human microvascular endothelial cell-conditioned medium were measured by enzyme-linked immunosorbent assays (ELISA) as previously described (22).

Corneal pocket assay. Slow-release EVA copolymer pellets containing 200 U of TNF-α with 20 μg each of control preimmune antibody, anti-IL-8 antibody, and anti-VEGF antibody were prepared and implanted in the corneas of 2- to

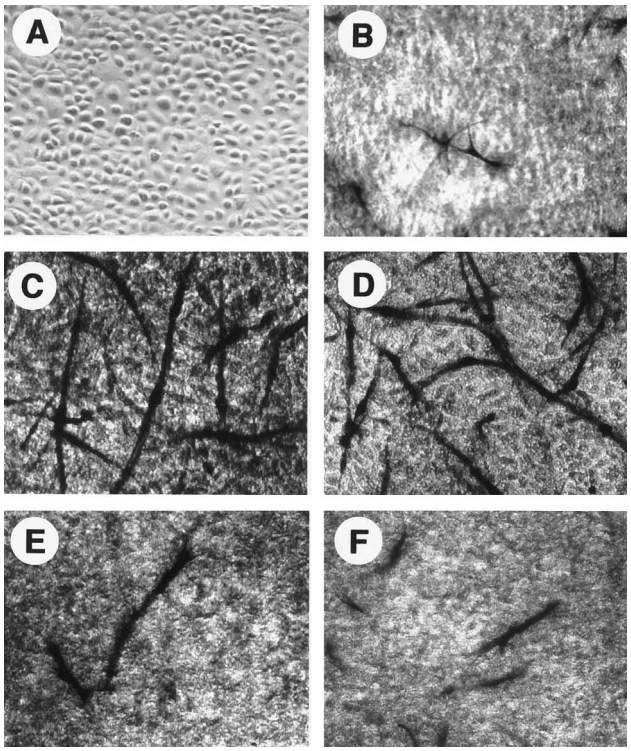


FIG. 2. Development of tube-like structures in human microvascular endothelial cells. Human microvascular endothelial cells were plated onto a collagen gel, and at confluence, cells assumed a cobblestone-like appearance (A). Vessel-like structures were analyzed 72 h after incubation in medium containing 1% serum (B) or after a 15-min treatment with TNF-α (100 U/ml) (C) or IL-8 (20 ng/ml) (D). Alternatively, cells were treated with TNF-α (100 U/ml) for 15 min and then incubated for an additional 72 h with an anti-IL-8 antibody (E) or with Sp1 antisense oligonucleotides (F). Prior to photography, the cells in panels B to F were fixed and stained briefly in modified May Gruenwald's solution to visualize tube-like structures. Magnification, ×348.

3-kg Japanese White rabbits, and the responses were evaluated as previously described (16). Five rabbits were used to assess the effect of each antibody.

RESULTS

Induction of IL-8, VEGF, bFGF, EGF/TGF-α, and their receptors by TNF-α. We previously demonstrated that the treatment of human microvascular endothelial cells with TNF-α enhanced production of the bFGF angiogenic factor (46) and that the treatment of human glioma cells with TNF-α induced production of another angiogenic factor, VEGF (56). IL-8 production in corneal endothelial and stromal cells was also enhanced in response to TNF-α (10). In this study, we assessed the expression of various angiogenic factors, including IL-8, VEGF, bFGF, and EGF/TGF-α, after TNF-α treatment by Northern blot analysis. The treatment of cells with 100 U of TNF-α per ml for 15 min followed by incubation in TNF-α-free medium for 3, 6, and 12 h enhanced mRNA levels of IL-8 by 11-, 5-, and 10-fold, respectively. Similarly, VEGF mRNA expression was increased by 6-, 8-, and 9-fold, respectively, and bFGF mRNA expression was increased by 4-, 10-, and 11-fold, respectively. In contrast, there was no change in EGF/TGF-α expression at any of these time points after TNF-α treatment (Fig. 1A).

Additionally, we analyzed IL-8, VEGF, and bFGF receptor expression by quantitative RT-PCR and EGF receptor mRNA levels by Northern blot analysis with TNF-α-treated endothelial cells (Fig. 1). Serially diluted receptor cDNAs were amplified by PCR, the radioactivities of the resulting products were plotted against template amounts, and then the quantitative ranges in which the reactions proceeded exponentially were determined (data not shown) (41, 44). We chose 100-ng templates for IL-8R and flt-1 mRNA analysis and 5-ng templates for flk-1 (VEGF receptor), flg (bFGF receptor), and β2-microglobulin mRNA analysis. Under these conditions, TNF-α stimulation for 3 h enhanced the expression of IL-8R, flt-1, flk-1, and flg by seven-, five-, seven-, and ninefold, respectively, in vascular endothelial cells. Moreover, for VEGF receptors, the rank order of mRNA abundance was flk-1 ≫ flt-1 (data not shown). The expression of EGF receptor was induced only slightly (1.5-fold higher) in TNF-α-treated cells. These data indicate that TNF-α resulted in increased levels of IL-8, VEGF, bFGF, and their cognate receptors in vascular endothelial cells.

Tubular morphogenesis of vascular endothelial cells after exposure to TNF-α. To examine the underlying mechanism(s) for TNF-α-induced angiogenesis, we developed an in vitro model system to assay tubular morphogenesis of vascular endothelial cells (45, 47). Human microvascular endothelial cells cultured on the surface of a three-dimensional type I collagen gel showed a cobblestone-like appearance in the absence of any angiogenic growth factor (Fig. 2A). Tube-like structures appeared on collagen gels when cells were treated with TNF-α (100 U/ml) or IL-8 (30 ng/ml), a condition under which no cytotoxic effects were observed (Fig. 2C and D), but they were minimal in the absence of exogenous factors (Fig. 2B). At the concentrations used, TNF-α and IL-8 induced tubular morphogenesis at comparable rates. We examined the effects of various doses of TNF-α on the formation of tube-like structures by vascular endothelial cells. The total length of tube-like structures was 0.90 ± 0.17 mm in the absence of treatment; the total lengths were 1.56 ± 0.13, 2.16 ± 0.30, 3.28 ± 0.25, 3.16 ± 0.34, and 1.43 ± 0.20 mm after cells were exposed to 1, 10, 100, 250, and 500 U of TNF-α per ml, respectively; and the total

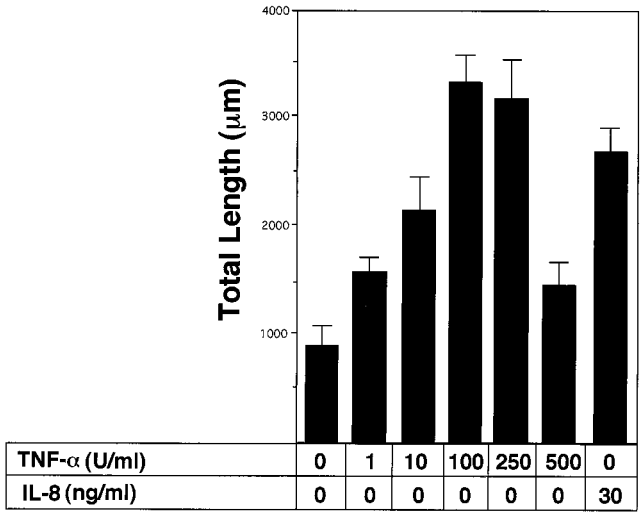
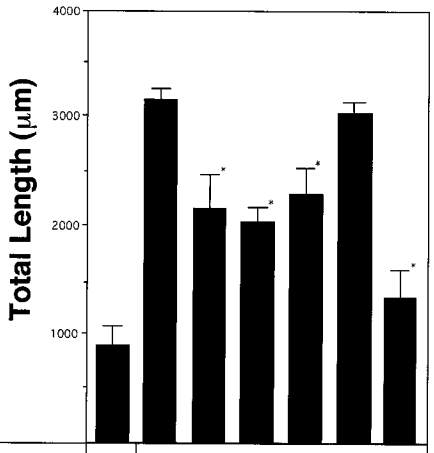


FIG. 3. Tube formation induced by various doses of TNF-α. Confluent endothelial cells were treated with 1 to 500 U of TNF-α per ml for 15 min, and the medium was replaced with M-199 medium containing 1% FBS. On the third day after treatment, tube formation was quantified. Phase-contrast microscopic pictures were then recorded with a still video camera recorder. The total length of tube-like structures was determined with an image analyzer. Eight random fields were measured, and the total length per field was calculated. Data are means ± standard deviations of the means (error bars) of triplicate experiments.



TNF-α (U/ml)	0	100					
anti-IL-8 Ab (μg/ml)	0	0	10	0	0	0	10
anti-VEGF Ab (μg/ml)	0	0	0	10	0	0	10
anti-bFGF Ab (μg/ml)	0	0	0	0	10	0	10
anti-EGF/TGF-α Ab (μg/ml)	0	0	0	0	0	10	0

FIG. 4. Inhibition of TNF-α-induced tube formation by antibodies against IL-8, VEGF, bFGF, and EGF/TGF-α. Cells were exposed to TNF-α (100 U/ml) for 15 min, and the medium was replaced with M-199 medium containing 1% FBS with or without antibodies (Ab) against IL-8, VEGF, bFGF, and EGF/TGF-α for 72 h. *, statistically significant difference ($P < 0.01$) compared with the value obtained in the absence of antibodies.

length was 2.69 ± 0.22 mm in the presence of IL-8 (Fig. 3). We used 100 U of TNF-α per ml in subsequent experiments.

Inhibition of TNF-α-induced tubular morphogenesis by anti-IL-8-, anti-VEGF-, and anti-bFGF-specific antibodies. We examined whether TNF-α-induced formation of tube-like structures is mediated through autocrine control by TNF-α-induced factors, such as IL-8, VEGF, and/or bFGF. Vascular endothelial cells were exposed to TNF-α (100 U/ml) for 15 min as described above and then were incubated in medium containing 1% serum with antibodies against IL-8, VEGF, and bFGF (10 μg/ml). An example of the results of this assay is shown in Fig. 2E. Treatment with anti-IL-8 antibody inhibited the TNF-α-induced development of tube-like structures. Quantitative analysis revealed that the TNF-α-induced increase in the total length of tube-like structures was significantly inhibited, by about 40 to 50% (Fig. 4), and coadministration of the three antibodies inhibited TNF-α-specific tube formation by more than 80% (Fig. 4). However, exogenous addition of anti-EGF/TGF-α antibody did not inhibit TNF-α-dependent tubular morphogenesis. Additionally, increasing the concentrations of antibodies to levels of >10 μg/ml did not induce a greater inhibitory effect (data not shown), suggesting that the employed antibody dose of 10 μg/ml was sufficient to antagonize the expressed cytokines. Thus, these data suggest that TNF-α-induced tubular morphogenesis is due to autocrine control by IL-8, VEGF, and bFGF.

Inhibition of IL-8, VEGF, and bFGF production by antisense oligonucleotides against Sp1, NF-κB, and c-Jun. To further examine the autocrine involvement of IL-8, VEGF, and bFGF in TNF-α-induced tubular morphogenesis, we assessed the effects of antisense oligonucleotides targeted against several transcription factors known to be responsible for their TNF-α-induced expression. The altered expression of cytokines through the introduction of cognate antisense oligonucleotides may be expected to modulate TNF-α-dependent tubular morphogenesis. The IL-8 gene promoter contains binding sites for AP-1 (Jun/Fos), NF–IL-6, and NF-κB (40), and the 5′-flanking regions of VEGF and bFGF contain several Sp1 binding sites as well as AP-1 binding sites (63, 67) (Fig. 5A). We previously demonstrated TNF-α induction of rapid activation of Sp1, NF-κB, and AP-1 in human microvascular endothelial cells (17, 23). Therefore, sense and antisense oligonucleotides against Sp1, NF-κB, and AP-1 were constructed; their effects were first confirmed by Western blot analysis (Fig. 5B). Whole-cell lysates were prepared from vascular endothelial cells that were cultured for 48 h with 20 μM either sense or antisense oligonucleotide directed against Sp1, NF-κB, or c-Jun. The cellular levels of Sp1, p65 (NF-κB), and c-Jun were all

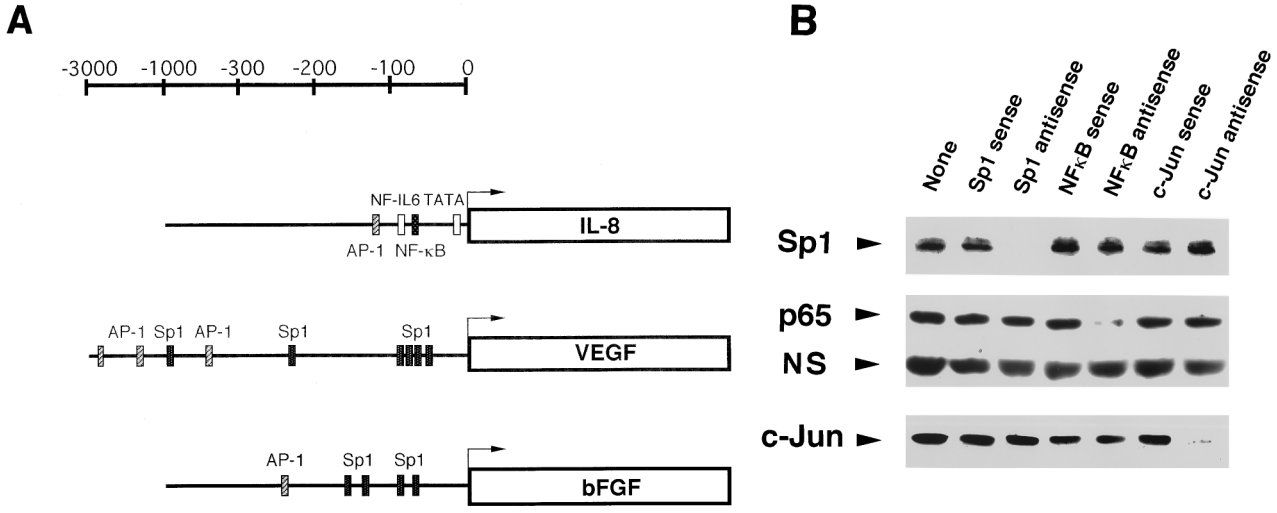
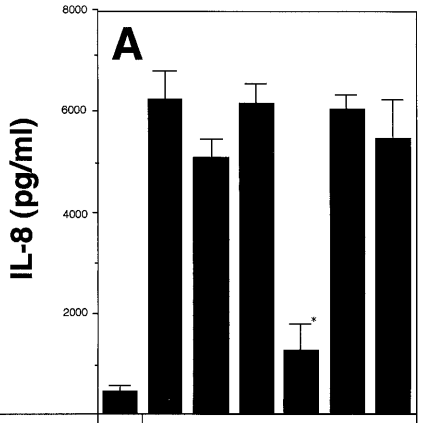
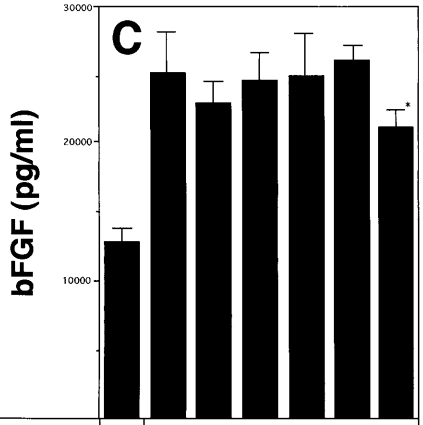


FIG. 5. Schemata of the 5′-flanking regions of the IL-8, VEGF, and bFGF genes (A) and effects of antisense oligonucleotides against Sp1, NF-κB, and c-Jun on their expression (B). (A) Binding sites for the Sp1, NF-κB, AP-1, and NF–IL-6 transcription factors in the 5′-flanking regions of the IL-8 (40), VEGF (67), and bFGF (63) genes are presented. (B) Endothelial cells were incubated in M-199 medium containing 10% FBS with 20 μM sense or antisense Sp1, NF-κB, or c-Jun oligonucleotide for 48 h. Cellular levels of Sp1, p65 (NF-κB), and c-Jun were assessed by fractionating whole-cell lysates on an SDS–10% polyacrylamide gel and immunoblotting with polyclonal anti-Sp1, anti-p65(NF-κB), and anti-c-Jun antibodies. NS, nonspecific.



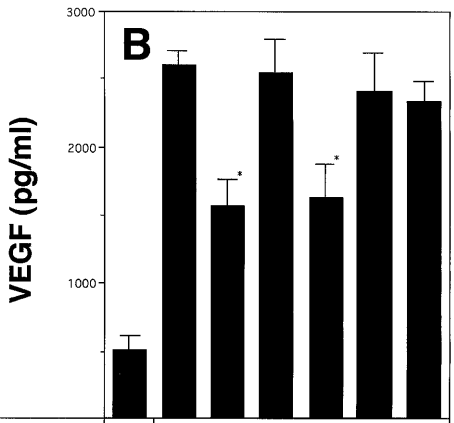
TNF-α (U/ml)	0	100					
Sp1 sense (μM)	0	20	0	0	0	0	0
Sp1 antisense (μM)	0	0	20	0	0	0	0
NFκB sense (μM)	0	0	0	20	0	0	0
NFκB antisense (μM)	0	0	0	0	20	0	0
c-Jun sense (μM)	0	0	0	0	0	20	0
c-Jun antisense (μM)	0	0	0	0	0	0	20



TNF-α (U/ml)	0	100					
Sp1 sense (μM)	0	20	0	0	0	0	0
Sp1 antisense (μM)	0	0	20	0	0	0	0
NFκB sense (μM)	0	0	0	20	0	0	0
NFκB antisense (μM)	0	0	0	0	20	0	0
c-Jun sense (μM)	0	0	0	0	0	20	0
c-Jun antisense (μM)	0	0	0	0	0	0	20

found to be specifically decreased by incubation with the corresponding antisense oligonucleotide, but not with the corresponding sense oligonucleotide (Fig. 5B).

To determine whether these transcription factors were specifically involved in TNF-α-induced IL-8, VEGF, and bFGF production, cytokine levels were measured in the medium or cellular fraction when endothelial cells were treated with Sp1, NF-κB, or c-Jun sense or antisense oligonucleotide (20 μM) for 48 h followed by exposure to TNF-α (100 U/ml) for 15 min. The medium was replaced with serum-free M-199 medium, and cells were incubated for an additional 48 h. The medium and cellular fractions were collected and subjected to cytokine ELISA. Treatment with TNF-α increased the levels of IL-8

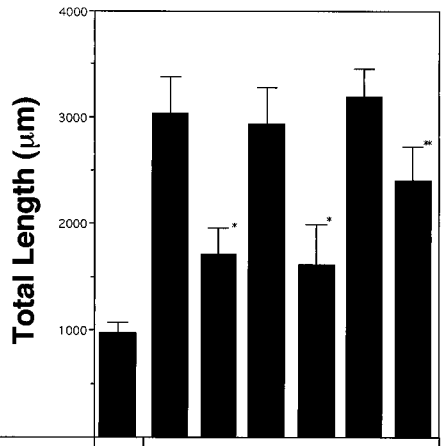


TNF-α (U/ml)	0	100					
Sp1 sense (μM)	0	20	0	0	0	0	0
Sp1 antisense (μM)	0	0	20	0	0	0	0
NFκB sense (μM)	0	0	0	20	0	0	0
NFκB antisense (μM)	0	0	0	0	20	0	0
c-Jun sense (μM)	0	0	0	0	0	20	0
c-Jun antisense (μM)	0	0	0	0	0	0	20

FIG. 6. Inhibition of IL-8, VEGF, and bFGF production by Sp1, NF-κB, and c-Jun antisense oligonucleotides. Endothelial cells were plated onto a 24-well plastic plate in M-199 medium containing 10% FBS. After cells were confluent, 20 μM sense or antisense Sp1, NF-κB, or c-Jun oligonucleotide was added to the medium and incubated for 48 h. Cells were treated with TNF-α (100 U/ml) for 15 min, and the medium was replaced with serum-free M-199 medium. After an additional 48-h incubation, the conditioned medium or cellular fraction was collected and IL-8, VEGF, and bFGF concentrations were determined by ELISA. *, statistically significant difference ($P < 0.01$) compared with the value obtained with the corresponding sense oligonucleotide.

and VEGF in the medium by 10- and 5-fold, respectively, and the level of bFGF in the cell fraction was also increased by 2-fold after TNF-α stimulation (Fig. 6). Administration of the NF-κB antisense oligonucleotide almost completely inhibited TNF-α-induced production of IL-8 ($P < 0.01$), but the Sp1 and c-Jun antisense oligonucleotides did not significantly affect IL-8 expression (Fig. 6A). The addition of both Sp1 and NF-κB antisense oligonucleotides inhibited TNF-α-dependent VEGF production by about 50%, but the c-Jun antisense oligonucleotide had only minimal effects, despite the presence of an AP-1 site in its promoter region (Fig. 6B). In contrast, there was about 30% inhibition of TNF-α-dependent bFGF production by the c-Jun antisense oligonucleotide; neither the NF-κB nor the Sp1 antisense oligonucleotide had any effect (Fig. 6C). In this assay, we assessed the cellular levels of bFGF (Fig. 6C), but secreted bFGF was also enhanced, albeit at lower levels, in TNF-α-treated cells (data not shown).

Inhibition of tubular morphogenesis in vascular endothelial cells by Sp1, NF-κB, and c-Jun antisense oligonucleotides. Three transcription factors, Sp1, NF-κB, and AP-1, appeared to be involved in TNF-α-induced production of the IL-8, VEGF, and bFGF angiogenic factors in human microvascular endothelial cells. We examined whether administration of antisense oligonucleotides directed against these three transcription factors modulated TNF-α-dependent tubular morphogenesis. Confluent cells on a type I collagen gel surface were incubated with 20 μM Sp1, NF-κB, or c-Jun sense or antisense oligonucleotide for 48 h and then treated with TNF-α (100 U/ml) for 15 min. After an additional 72-h incubation, TNF-



TNF-α (U/ml)	0	100					
Sp1 sense (μM)	0	20	0	0	0	0	0
Sp1 antisense (μM)	0	0	20	0	0	0	0
NFκB sense (μM)	0	0	0	20	0	0	0
NFκB antisense (μM)	0	0	0	0	20	0	0
c-Jun sense (μM)	0	0	0	0	0	20	0
c-Jun antisense (μM)	0	0	0	0	0	0	20

FIG. 7. Inhibition of TNF-α-induced tube formation by Sp1, NF-κB, and c-Jun antisense oligonucleotides. Endothelial cells were plated onto a collagen gel in M-199 medium containing 10% FBS and were grown until nearly confluent. Sense or antisense Sp1, NF-κB, or c-Jun oligonucleotide (20 μM) was added to the medium, and the mixture was incubated for 48 h. After 15 min of TNF-α (100 U/ml) stimulation, the medium was replaced with M-199 medium containing 1% FBS for an additional 72-h incubation. Tube formation was then quantified. Statistically significant differences (*, $P < 0.01$; **, $P < 0.02$) compared with the value obtained by using the corresponding sense oligonucleotide are indicated.

α-induced formations of tube-like structures were assessed. An example of the effect of the Sp1 antisense oligonucleotide is depicted in Fig. 2F. Tubular morphogenesis was quantified by measuring the total tube length developed on a collagen gel. Sp1, NF-κB, and c-Jun sense oligonucleotides did not inhibit TNF-α-induced tube formation (Fig. 7). However, pretreatment with the Sp1 and NF-κB antisense oligonucleotides blocked TNF-α-induced tube formation by about 60% (Fig. 7) and the c-Jun antisense oligonucleotide inhibited tube formation by about 30%. Therefore, Sp1, NF-κB, and AP-1 all appear to be involved in TNF-α-dependent tubular morphogenesis by human microvascular endothelial cells. Moreover, Sp1 and NF-κB appear to be more closely coupled with this TNF-α-induced process than is AP-1.

Inhibition of TNF-α-induced angiogenesis by anti-IL-8 and anti-VEGF antibodies in vivo. To address the possible participation of IL-8 and VEGF in TNF-α-induced angiogenesis in vivo, we implanted EVA pellets, impregnated with TNF-α with or without anti-IL-8 or anti-VEGF antibody, into rabbit corneas. TNF-α (200 U) with control preimmune antibody (20 μg) elicited an angiogenic response (Fig. 8B), but simultaneous administration of TNF-α (200 U) and anti-IL-8 antibody (20 μg) resulted in a greatly attenuated angiogenic response (Fig. 8C). An anti-VEGF antibody also inhibited TNF-α-induced angiogenesis, but to a slightly lower degree than that induced by the anti-IL-8 antibody (Fig. 8D). Since available antibodies against bFGF failed to antagonize rabbit bFGF, it was not possible to examine whether bFGF is involved in TNF-α-induced neovascularization in the rabbit cornea.

DISCUSSION

TNF-α has been presented as a potent angiogenesis-promoting factor in in vivo angiogenesis models with the cornea and chorioallantoic membrane (15, 29), in an in vivo model with reconstituted basement membrane (50), and in an in vitro system with human microvascular endothelial cells (64). Leek et al. (28) have reported that tumor-associated macrophage infiltration is closely correlated with tumor angiogenesis in patients with invasive breast cancer, suggesting an involvement of macrophage-secreted TNF-α in angiogenesis in vivo. Consistent with these data, our present study indicated that short exposure to TNF-α (15 min) induced tube-like structures in an in vitro collagen gel assay and induced angiogenesis in rabbit corneas. We questioned whether TNF-α itself was directly involved in angiogenesis or, alternatively, whether TNF-α-activated angiogenic factors mediated this process. We found that (i) TNF-α enhanced the production of IL-8, VEGF, and bFGF, all of which are potent angiogenic factors. Moreover, antibodies against IL-8, VEGF, and bFGF were able to block TNF-α-dependent tubular morphogenesis. (ii) Coadministration of an anti-IL-8 or anti-VEGF antibody blocked TNF-α-induced development of capillary networks in a cornea assay. (iii) Coadministration of Sp1, NF-κB, and c-Jun antisense oligonucleotides, which blocked TNF-α-dependent induction of IL-8, VEGF, and bFGF, also interfered with TNF-α-induced tubular morphogenesis. (iv) TNF-α enhanced expression of the IL-8, VEGF, and bFGF receptors (IL-8R, flt-1, flk-1, and flg, respectively). Since TNF-α enhances the expression of VEGF, IL-8, bFGF, and their receptors in vascular endothelial cells, stromal cells and cancer cells, this process is likely important in promoting macrophage-induced neovascularization in pathologic areas (Fig. 9). In the in vitro model, the inhibition of TNF-α-induced tubular morphogenesis by coadministration of antibodies against IL-8, VEGF, and bFGF was not complete (Fig. 4), suggesting that other factors are also involved. However, our data clearly indicate that these three cytokines play a major role as secondary mediators in TNF-α-induced angiogenesis through paracrine and/or autocrine control (Fig. 9).

As discussed earlier, TNF-α also has antiangiogenic properties in in vitro models with bovine vascular endothelial cells and human umbilical endothelial cells (58, 62) and in an in vivo corneal assay system (3). In the in vitro system, an exposure to TNF-α (100 U/ml) of >6 h inhibited all angiogenesis-related processes, with inhibition of plasminogen activator gene expression, cell migration, and tubular morphogenesis on collagen gels and induction of dramatic changes in cellular β-actin levels and cell morphology from cobblestone-like to spindle-like microvascular endothelial cells (25, 36, 37). In addition, Fajardo et al. (11) reported this to be a dose-dependent effect, with lower TNF-α concentrations promoting angiogenesis and higher concentrations inhibiting this process. The diverse effects of TNF-α may rely on a balance of the angiogenic and antiangiogenic factors, stimulated by TNF-α. Positive factors induced by TNF-α in vascular endothelial cells include IL-8 and VEGF (this study), bFGF (46) (this study), collagenase (36, 46), prostaglandins (11), platelet-activating factor (39), and B61 (49), while TNF-α-induced production of plasminogen activator inhibitor-1 (37, 59) and downregulation of integrin αVβ5 (6) and tissue-type plasminogen activator (37, 59) inhibit angiogenesis. This balance may be modified, depending on the local concentration or duration of exposure to TNF-α, resulting in modulation of TNF-α-induced angiogenesis. Further investigation is required to understand the underlying mechanism by which the angiogenesis "switch" is controlled by TNF-α.

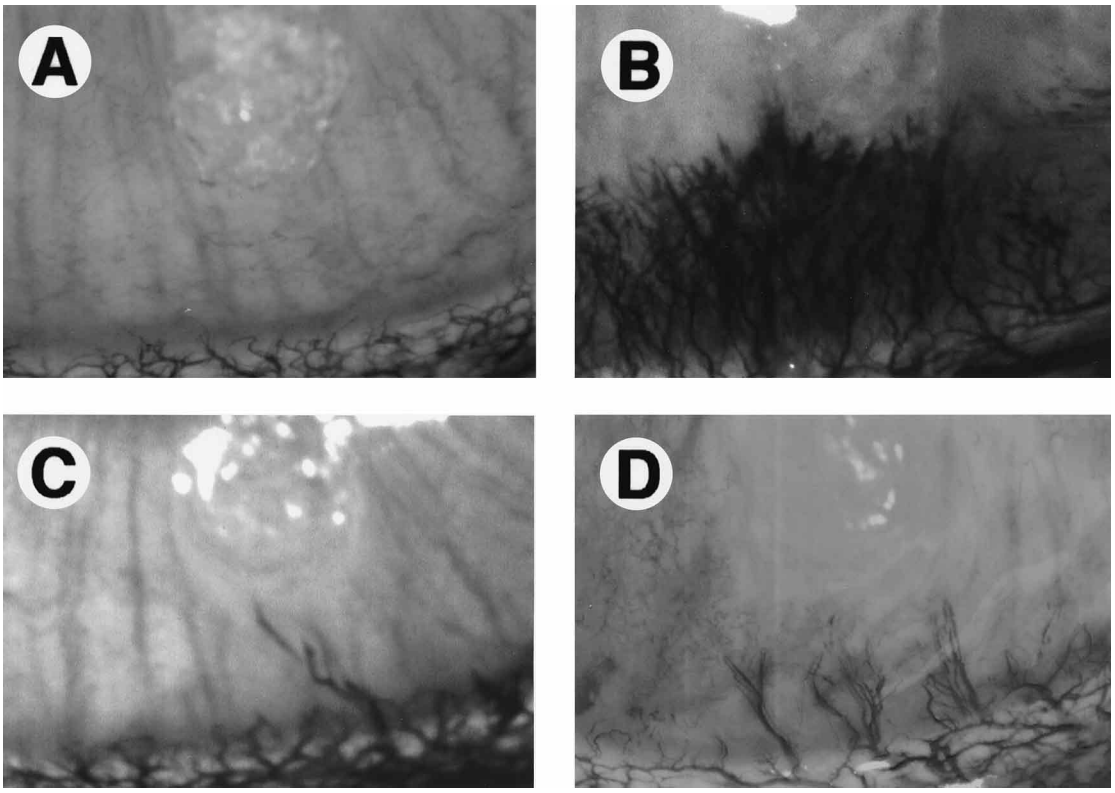


FIG. 8. Inhibition of TNF-α-induced angiogenesis by an anti-IL-8 or anti-VEGF antibody in the rabbit cornea. Shown are rabbit corneas 10 days after implantation with EVA pellets containing phosphate-buffered saline alone (A), TNF-α (200 U) with control antibody (20 μg) (B), TNF-α (200 U) with polyclonal anti-IL-8 antibody (20 μg) (C), and TNF-α (200 U) with anti-VEGF antibody (20 μg) (D).

TNF-α induces the production of reactive oxygen species such as H_2O_2 (33). H_2O_2 has been shown to stimulate the expression and DNA binding activity of the NF-κB transcription factor in endothelial cells (60). TNF-α-induced activation of NF-κB appears to be mediated in a reactive oxygen species-dependent manner (60). NF-κB is involved in angiogenesis (64, 65), and H_2O_2 itself stimulates the formation of tube-like structures by microvascular endothelial cells (64). In our study, coadministration of NF-κB antisense oligonucleotide resulted in partial inhibition of TNF-α-induced angiogenesis. The observed partial inhibition by an NF-κB antisense oligonucleotide may be due to its inhibitory effect on IL-8, VEGF, and flk-1 production; both IL-8 expression and VEGF expression were inhibited by the NF-κB antisense oligonucleotide (Fig. 5 to 7). Since the 5′-flanking region of the VEGF promoter has no typical NF-κB binding motif (Fig. 5A), it was surprising that the NF-κB antisense oligonucleotide inhibited VEGF production. Chiao and colleagues reported that the VEGF promoter region may contain an NF-κB-like binding site, suggesting NF-κB participation in the control of VEGF gene expression (4a). However, as the inhibition by the NF-κB antisense oligonucleotide of TNF-α-dependent tubular morphogenesis was partial, this suggests the involvement of other factors in TNF-α-induced angiogenesis. In fact, our present study indicates the involvement of the Sp1 and c-Jun transcription factors in this process.

Although Sp1 is activated by TNF-α (17, 23) but not by H_2O_2 (64) in endothelial cells, the specific Sp1 antisense oligonucleotide also inhibited TNF-α-induced angiogenesis. Sp1 has a DNA binding domain composed of three zinc fingers and binds to the GC box [consensus sequence, (G/T)GGGCGG (G/A)(G/A)(C/T)], activating a variety of cellular and viral promoters in a differentially regulated manner (57). The Sp1 antisense oligonucleotide inhibited VEGF expression and may be the mechanism by which TNF-α-induced angiogenesis is affected. Moreover, we recently demonstrated that enhancement of VEGF gene expression by TNF-α requires clustered Sp1 sites on the VEGF promoter (56).

The NF-κB and Sp1 antisense oligonucleotides were equally

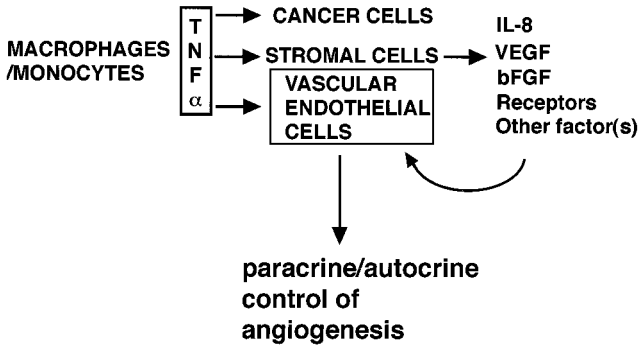


FIG. 9. Presumed mechanism of neovascularization induced by macrophage-involving pathologic conditions. Macrophages/monocytes migrate into pathologic areas and release TNF-α, resulting in enhanced expression of IL-8, VEGF, bFGF, and their receptors in vascular endothelial cells. TNF-α also enhances the production of IL-8, VEGF, and bFGF in stromal and tumor cells. Upregulated receptors include IL-8R, flt-1, flk-1 and flg. These three potent angiogenic factors likely promote neovascularization. Consistent with this model, macrophages/monocytes stimulate tumor cells to secrete VEGF (5) and there is active secretion of TNF-α, VEGF, and bFGF in the tumor microenvironment (30, 54).

efficient inhibitors of TNF-α-induced tube formation. The NF-κB and Sp1 antisense oligonucleotide-dependent inhibition of TNF-α-induced tube formation might be mediated not only through interfering with transcription of IL-8, VEGF, and bFGF but also through interfering with their receptors. The promoter region of flk-1 contains several potential binding sites for Sp1 and NF-κB (51), suggesting that these transcription factors regulate expression of the VEGF receptor as well as its cognate ligand. Functional cooperation between Sp1 and NF-κB has previously been reported (31, 52), and these two transcription factors may play a synergistic role in TNF-α induction of VEGF and transcription of its cognate receptor.

The AP-1 (Jun/Fos) antisense oligonucleotide induced weaker but significant inhibition of TNF-α-dependent tubular morphogenesis. AP-1 sites are ubiquitously located on the promoters of various angiogenesis-related genes, including the IL-8, VEGF, and bFGF promoters (Fig. 5A). Asakuno et al. (2) reported that overexpression of a transfected c-*fos* gene induced very high angiogenesis in the rat brain, suggesting an involvement of AP-1 in in vivo angiogenesis. In comparison with NF-κB and Sp1 antisense oligonucleotides, introduction of the c-Jun antisense oligonucleotide did not significantly lower IL-8 and VEGF production. However, it did reduce the TNF-α-induced bFGF levels by 30%. Partial inhibition of bFGF production by the c-Jun antisense oligonucleotide suggests that the AP-1 pathway has some direct involvement in tubular morphogenesis by microvascular endothelial cells. Alternatively, the introduction of the c-Jun antisense oligonucleotide may modulate other angiogenesis-related genes, such as metalloproteinases and plasminogen activators. It remains to be determined how closely AP-1 is coupled with angiogenesis under normal and pathologic conditions.

We found that TNF-α enhanced expression of angiogenic ligands, such as IL-8, VEGF, and bFGF, and their cognate receptors, such as IL-8R, flt-1, flk-1, and flg in vascular endothelial cells, suggesting an autocrine loop for angiogenesis. Okamura et al. (46) also reported enhanced expression and secretion of bFGF, collagenase, and IL-6 from TNF-α-treated human microvascular endothelial cells. Additionally, we demonstrated that the treatment of human glioma cells with TNF-α induced VEGF expression (56). In contrast, Elner et al. (10) demonstrated that TNF-α significantly increased IL-8 levels in corneal endothelial and stromal cells, suggesting that the effect of TNF-α arises from the direct stimulation of stromal and endothelial cells. IL-8, a potent neutrophil chemoattractant, induces angiogenesis in vitro as well as in vivo (24, 64, 68). These observations may be relevant to the angiogenesis observed in inflammation, solid tumors, and other pathologic conditions (13, 18). If TNF-α enhances the production of VEGF, IL-8, and other angiogenic factors in stromal cells and other cells adjacent to microvessels, the angiogenesis pathway is also likely to be under paracrine control in vivo (Fig. 9).

In conclusion, TNF-α-dependent angiogenesis appears to be mediated through IL-8, VEGF, and bFGF as secondary mediators. This is the first report to direct demonstrate the involvement of the Sp1, NF-κB, and AP-1 transcription factors in TNF-α-induced angiogenesis. Antibodies directed against these growth factors, as well as Sp1 and NF-κB antisense oligonucleotides, may be of therapeutic value in the treatment of angiogenesis-related diseases, such as solid tumors, rheumatoid arthritis, psoriasis, and diabetic retinopathy.

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