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# Involvement of the Transcription Factor NF-κB in Tubular Morphogenesis of Human Microvascular Endothelial Cells by Oxidative Stress

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**Oxygen radicals are induced under various pathologic conditions associated with neovascularization. Oxygen radicals modulate angiogenesis in cultured human microvascular endothelial cells by an unknown mechanism. Treatment of human microvascular endothelial cells for 15 min with 0.1 to 0.5 mM hydrogen peroxide ($H_2O_2$) or 100 U of tumor necrosis factor alpha per ml induced tubular morphogenesis in type I collagen gels. Gel shift assays with nuclear extracts demonstrated that $H_2O_2$ increases the binding activities of two transcription factors, NF-κB and AP-1, but not of Sp1. Tumor necrosis factor alpha increased the binding activities of all three factors. A supershift assay with specific antibodies against JunB, JunD, and c-Jun (Jun family) showed that the antibody against c-Jun supershifted the AP-1 complex after $H_2O_2$ treatment. Coadministration of the antisense sequence of NF-κB inhibited $H_2O_2$-dependent tubular morphogenesis, and the antisense c-Jun oligonucleotide caused partial inhibition. The angiogenic factor responsible for $H_2O_2$-induced tubular morphogenesis was examined. Cellular mRNA levels of vascular endothelial growth factor and interleukin-8 (IL-8), but not those of transforming growth factor α, were increased after treatment with 0.5 mM $H_2O_2$. Coadministration of anti-IL-8 antibody inhibited tubular morphogenesis enhanced by $H_2O_2$, and IL-8 itself also enhanced the formation of tube-like structures. Treatment with antisense NF-κB oligonucleotide completely blocked $H_2O_2$-dependent IL-8 production by endothelial cells. The tubular morphogenesis of vascular endothelial cells after treatment with oxidative stimuli and its possible association with NF-κB and IL-8, is examined.**

Angiogenesis is an important process in inflammation, the enlargement of solid tumors, and other pathologic phenomena (11). It is thought to be regulated by various growth factors and cytokines, including epidermal growth factor (EGF)/transforming growth factor α (TGF-α), TGF-β, angiogenin, tumor necrosis factor alpha (TNF-α), acidic fibroblast growth factor, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF)/vascular permeability factor, and interleukin-8 (IL-8). However, the initiation of angiogenesis still remains unclear. Sometimes induction of angiogenic factors is triggered by various stresses; for instance, hypoxic stress induces angiogenic signaling in human glioma cells (57) and in retinas in a mouse model of retinopathy (50). Of the putative angiogenic factors, expression of the VEGF gene is highly susceptible to hypoxic stress (9, 13, 23, 28, 50, 57). Analysis of the VEGF gene promoter demonstrates several potential binding sites for the transcription factors Sp1, AP-1, and AP-2 (59). The hypoxia-induced expression of the VEGF gene is mediated through activation of AP-1 (13), hypoxia-responsible elements (36), c-*src* (41), and VEGF mRNA stability (10) in various cell types.

Reactive oxygen intermediates (ROIs) are generated under various physiologic and pathologic conditions, including inflammation, ischemia and reperfusion, sepsis, and UV irradiation (48). Major ROIs are the superoxide anion ($O_2^-$), hydroxyl radical ($^{\cdot}OH$), and $H_2O_2$. Elevated intracellular ROI levels are referred to as oxidative stress. The vascular endothelium is one of the prime targets for oxidative stress in a variety of inflammatory conditions, possibly mediated by activated phagocytes that release oxygen radicals (56, 61). Although ROIs have cytotoxic effects under oxidative stress, evidence has shown that oxidants mediate some biologic responses (42, 56). Furthermore, several transcription factors often use $H_2O_2$ as a second messenger of their activation (2, 35, 54, 55).

NF-κB is an inducible transcription factor that was originally identified as a heterodimeric complex consisting of a 50-kDa subunit (originally called p50 and now designated NFKB1) and a 65-kDa subunit (originally called p65 and now designated RelA) (4, 12). It is thought to play a central role in the regulation of a number of immune and inflammatory response genes (14, 29) and is the first eukaryotic transcription factor shown to respond directly to oxidative stress (55). There is a possibility that these transcription factors regulate the initiation of angiogenesis; however, no direct evidence has been shown.

To investigate the mechanism of angiogenesis, we have established in vitro angiogenesis models. In these models with bovine aortic endothelial cells, cell migration or tubular morphogenesis is potentially stimulated by bFGF (1, 37, 39, 51) or VEGF (5, 30). In contrast, cell migration or tubular morphogenesis in human microvascular endothelial cells is highly responsive to TGF-α/EGF (34, 44, 47, 53) and to VEGF and IL-8

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(62). IL-8 is another angiogenic factor that is produced at high levels in monocytes, macrophages (33), and human brain tumors (22, 38, 60, 62). IL-8 induces cell migration of human umbilical endothelial cells, angiogenesis in rat corneas (25), and tubular morphogenesis by microvascular endothelial cells (62). Recent studies demonstrate that the IL-8 gene promoter region contains binding sites for AP-1 and NF-κB (32, 40, 46). Moreover, expression of the IL-8 gene is induced by oxidative stress and the induction is suppressed by various antioxidants (6, 7).

We examined whether oxidative stress induced the formation of tube-like structures in human microvascular endothelial cells, and which transcription or angiogenic factor was responsible for oxidative stress-induced angiogenesis. In this study, we demonstrated the involvement of the transcription factor NF-κB in $H_2O_2$ induction of tubular morphogenesis and also its associated effect on IL-8 production.

## MATERIALS AND METHODS

**Materials.** IL-8, human IL-8 cDNA, and anti-IL-8 antibody were previously described (24, 33). Human VEGF cDNA was obtained from H. A. Weich (Biotechnologische Forschung, Braunschweig, Germany), anti-VEGF antibody was from R & D Systems (Minneapolis, Minn.), TNF-α was from Mochida Pharmaceutical Co. (Tokyo, Japan), TGF-α was from Pepro Tech Inc., and human TGF-α cDNA was from R. Derynck (Genentech, Inc., South San Francisco, Calif.). Antibodies against c-Fos, Fra-1, JunB, JunD, and c-Jun were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Polyclonal anti-p65 antiserum was generated by multiple immunizations of a New Zealand White rabbit with synthesized peptides, as described previously (16, 17). Anti-p65 antibody was affinity purified with synthetic peptides. [$\gamma$-$^{32}$P]dATP, [$\alpha$-$^{32}$P]UTP, and [$\alpha$-$^{32}$P]dCTP were purchased from Amersham (Little Chalfont, United Kingdom). AP-1, Sp1, and NF-κB consensus sequences were synthesized with a DNA synthesizer (Applied Biosystems Inc.). The sequences of the oligonucleotides for AP-1, Sp1, and NF-κB are as follows: for AP-1, 5′-TCGAGCTGACT CATCGCTGACTCATC-3′ and 3′-CGACTGAGTAGCGACTGAGTAGCTA G-5′; for Sp1, 5′-TCGATCGGGGCGGGGCGATCGGGGCGGGGCGA-3′ and 3′-AGCCCCGCCCCGCTAGCCCCGCCCCGCTCTAG-5′; and for NF-κB, 5′-TCGAAGGGGACTTTCCCAAGGGGACTTTCCCA-3′ and 3′-TCCC CTGAAAGGGTTCCCCTGAAAGGGTCTAG-5′.

**Antisense oligonucleotides.** The antisense and sense phosphorothioate analogs of the oligonucleotides to the 5′ end of the NF-κB and c-*jun* genes, including the ATG initiation codon (30-mer), were synthesized as previously described (31). The sequences are as follows: for NF-κB, 5′-ACCCCGGCCATGGACGAACT GTTCCCCCTC-3′ (sense) and 5′-GAGGGGGAACAGTTCGTCCCATGGCC GGGGT-3′ (antisense); for c-Jun, 5′-ACGTGAAGTGACGGACTGTTCTAT GACTGC-3′ (sense) and 5′-GCAGTCATAGAACAGTCCGTCACTTCACGT -3′ (antisense).

**Human microvascular endothelial cells.** Human microvascular endothelial cells were isolated from surgically removed omental tissue as previously described (34, 44, 47, 53, 62).

**Quantitative analysis of tube formation in human microvascular endothelial cells in type I collagen gel.** Human microvascular endothelial cells were plated onto the surface of type I collagen gel in medium 199 containing 10% fetal bovine serum (FBS). When the cells reached confluence, the medium was replaced with medium 199 containing 1% FBS with or without factors and the cells were incubated for an additional 3 days. 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 the tube-like structures per field was measured with a Cosmozone image analyzer (Nikon, Tokyo, Japan) as previously described (44, 47, 53). Eight random fields per dish were measured, and the total length per field was calculated.

**Northern (RNA) blot analysis.** Human microvascular endothelial cells were grown in medium 199 containing 10% FBS, followed by serum-free medium 199 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, and 0.1 M β-mercaptoethanol. Total RNA was extracted as previously described (1, 15, 21). The 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$^2$ by using a FLUO-LINK (Viler Lourmat, Marne-La-Vallée, France). The membrane was hybridized to $^{32}$P-labeled DNA probes in Hybrisol (Oncor Inc., Gaithersburg, Md.) at 42°C for 24 h and then washed twice at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS), once in 1× SSC and 0.1% SDS, and once more in 0.2× SSC and 0.1% SDS. The mRNA levels were quantified by densitometry with a Fujix BAS 2000 bioimage analyzer (Fuji Photo Film Co.).

**Nuclear extract preparation.** Cells were washed with ice-cold phosphate-buffered saline, scraped from the plate with a rubber spatula, and suspended in buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride [15, 21]). The cells were allowed to swell on ice for 15 min; then Nonidet P-40 was added to a final concentration of 0.5%. After centrifugation, the pellet was washed once in buffer A. The nuclear pellet was resuspended in ice-cold buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg of pepstatin per ml, and 1 μg of leupeptin per ml) and swelled on ice for 30 min. The nuclear extract was clarified by centrifugation at 12,000 × *g* for 5 min. The supernatant was frozen at −80°C.

**Gel shift assay.** Nuclear extracts (4 μg of protein) were incubated in a 20-μl reaction mixture containing 10 mM HEPES (pH 7.9), 4% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 0.1 μg of bovine serum albumin, 1 μg each of poly (dI-dC) and 100× cold oligonucleotide as described with modifications (15, 21). The $^{32}$P-labeled oligonucleotide (2 μl) was added to the reaction mixture and incubated for 30 min at 25°C. In the supershift assay, antibodies (1 μg) were added after the binding reaction and the mixtures were incubated for 2 h at 4°C. Then 2 μl of 10× agarose loading buffer dye (50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was added, and the samples were electrophoresed on a 4% polyacrylamide gel (polyacrylamide/bisacrylamide ratio, 80:1) at 4°C with TGE (25 mM Tris base, 190 mM glycine, 1 mM EDTA) (pH 8.5). The gel was directly analyzed by autoradiography.

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

**ELISA of IL-8.** The concentration of IL-8 in the conditioned medium of the human microvascular endothelial cells was measured by enzyme-linked immunosorbent assay (ELISA) as previously described (20).

## RESULTS

**Tubular morphogenesis of microvascular endothelial cells after exposure to $H_2O_2$ and TNF-α.** Human microvascular endothelial cells cultured on the surface of three-dimensional type I collagen gel have a cobblestone-like appearance in the absence of any angiogenic growth factor, however, they form tube-like structures when treated with EGF or TGF-α (44, 47, 53).

Tubular formation of human microvascular endothelial cells occurred in type I collagen gels in the presence of 30 ng of TGF-α per ml (Fig. 1B). We examined whether oxidative stress from $H_2O_2$ or an angiogenic cytokine, TNF-α, could enhance the tubular morphogenesis of human microvascular endothelial cells. After the cells became confluent on type I collagen gel, they were treated with 0.5 mM $H_2O_2$ or 100 U of TNF-α per ml for 15 min. After a 3-day incubation, tube-like structures developed to a much greater extent when the cells were treated with $H_2O_2$ or TNF-α than in the absence of any treatment. $H_2O_2$ and TNF-α appeared to induce tubular morphogenesis at rates similar to those of TGF-α (Fig. 1B, C, and D). The total length of the tube-like structures was 0.80 ± 0.16 mm in the absence of treatment; 1.73 ± 0.05 mm, 2.86 ± 0.09 mm, and 3.57 ± 0.56 mm after the cells were exposed to 0.01 mM, 0.1 mM, and 0.5 mM $H_2O_2$, respectively; and 3.14 ± 0.40 mm after exposure to TNF-α (Fig. 2).

**Activation of DNA-binding proteins Sp1, AP-1, and NF-κB by $H_2O_2$ or TNF-α.** We previously demonstrated that TNF-α induces the rapid activation of AP-1, Sp1, and NF-κB in human microvascular endothelial cells (15, 21). Using a gel mobility shift assay (Fig. 3), we attempted to determine if specific transcription factors were responsible for the $H_2O_2$- or TNF-α-induced tubular morphogenesis. The binding activity of AP-1 and NF-κB increased 1 h after treatment with 0.5 mM $H_2O_2$ for 15 min, while Sp1 activation was not induced by oxidative stress. The $H_2O_2$ activation of AP-1 and NF-κB continued at 3 and 6 h after oxidative stress. AP-1 consists of a Fos-Jun heterodimer and a Jun homodimer. To identify the individual components of the induced AP-1 binding complex in

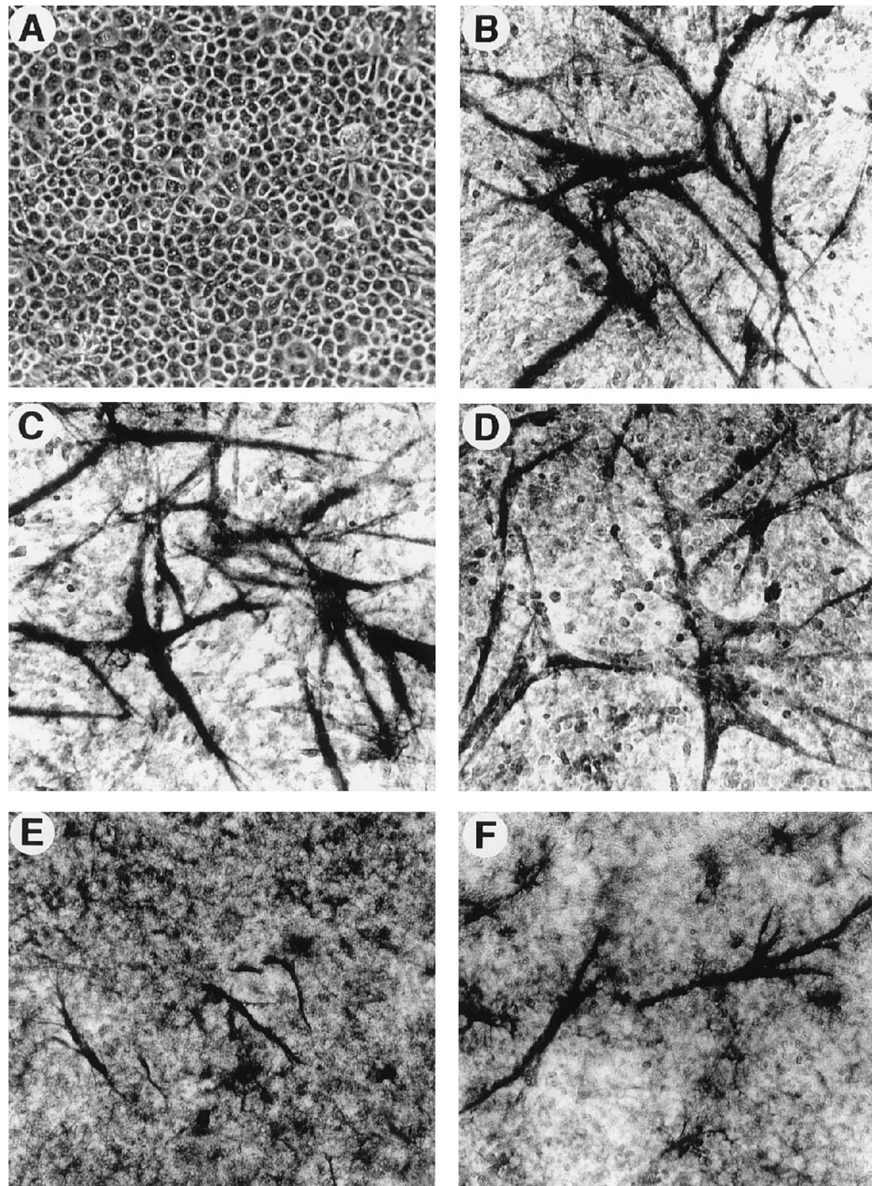



FIG. 1. Development of tube-like structures in human microvascular endothelial cells. Human microvascular endothelial cells were plated onto collagen gel when they were nearly confluent. The cells assumed a cobblestone-like appearance (A). Vessel-like structures appeared after a 3-day incubation in medium containing 1% serum with 30 ng of TGF-α per ml (B) and after 15 min of exposure to 100 U of TNF-α per ml (C) or 0.5 mM $H_2O_2$ (D). $H_2O_2$-induced tubular morphogenesis is inhibited by NF-κB antisense oligonucleotides (E) and anti-IL-8 antibody (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, ×400.

$H_2O_2$-treated endothelial cells, a supershift assay with specific antibodies against JunB, JunD, and c-Jun was performed (Fig. 4). Administration of antibody against c-Jun resulted in a supershift of AP-1 binding complex in both $H_2O_2$-treated and untreated endothelial cells, indicating that c-Jun is a component of these binding complexes. However, antibodies against JunB and JunD did not produce a supershift complex. Western blot analysis with specific antibodies against NF-κB or AP-1 was performed to examine the translocation of NF-κB and the levels of Fos family (c-Fos and Fra-1) and Jun family (c-Jun, JunB, and JunD) members in the nucleus (Fig. 5). The amount of NF-κB (p65) in the nuclear extract was increased in the cells treated with 0.5 mM $H_2O_2$ and 100 U of TNF-α per ml. The protein levels of Fra-1 and JunB were increased at 30 min and 1 h after $H_2O_2$ treatment but decreased at 3 and 6 h. Those of c-Fos and JunD seemed to be constant. The amount of c-Jun was transiently enhanced at 30 min after $H_2O_2$ stress and was constant between 1 and 6 h. The amount of Fos and Jun family proteins seems to have no correlation with the binding activity of AP-1.

**Inhibition of tubular morphogenesis in human microvascular endothelial cells by the antisense oligonucleotides of NF-κB and c-Jun.** $H_2O_2$ activated two transcription factors, NF-κB and AP-1, in microvascular endothelial cells. To determine whether the $H_2O_2$-induced angiogenesis in the model system was coupled with these transcription factors, the effect of antisense oligonucleotides of NF-κB or c-Jun was investigated and confirmed by Western blot analysis and gel shift

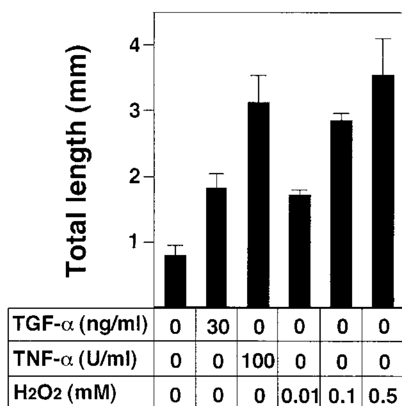



FIG. 2. Tube formation induced by TNF-α and $H_2O_2$. Confluent endothelial cells were treated with 0.01 to 0.5 mM $H_2O_2$ or 100 U of TNF-α per ml for 15 min and the medium was replaced with medium 199 containing 1% FBS. On the third day from the treatment, tube formation was quantified.

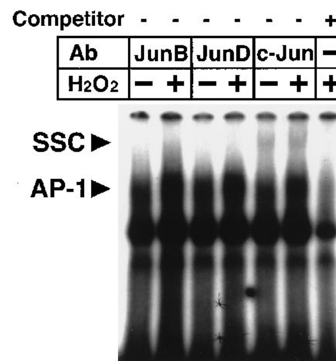



FIG. 4. Identification of the components of the AP-1 binding complex. Endothelial cells were incubated in serum-free medium 199 for 24 h and exposed to $H_2O_2$ (0.5 mM) for 15 min. After an additional 6-h incubation, the cells were harvested and nuclear extracts were prepared. The extracts were incubated with $^{32}$P-labeled oligonucleotides of AP-1 for 30 min at 25°C, and then antibody (Ab) against JunB, JunD, or c-Jun was added. The mixtures were incubated for 2 h at 4°C and resolved by gel electrophoresis. A 100-fold excess of the unlabeled oligonucleotides was added for the competition. The arrows indicate the retarded DNA-protein complexes of AP-1 and supershifted complex (SSC).

assay (Fig. 6). In the Western blot analysis, whole-cell lysates were prepared from microvascular endothelial cells that were cultured for 48 h with 20 μM sense or antisense oligonucleotides of NF-κB or c-Jun. The lysates were fractionated by SDS–10% PAGE. The cellular levels of NF-κB and c-Jun were found to be decreased with each antisense oligonucleotide but not with each sense oligonucleotide (Fig. 6A). In the gel shift assay, after a 48-h incubation with 20 μM sense or antisense oligonucleotides of NF-κB or c-Jun, the cells were treated with 0.5 mM $H_2O_2$ for 15 min and the medium was replaced. After an additional 6-h incubation, the cells were harvested and nuclear extracts were prepared. The binding activities of NF-κB and AP-1 were almost completely inhibited by each antisense oligonucleotide (Fig. 6B).

Modulation of the $H_2O_2$-dependent angiogenesis in the model system was examined with sense or antisense oligonucleotides. When the cells were confluent on the type I collagen gel surface, 20 μM sense or antisense oligonucleotides of NF-κB or c-Jun were placed in the medium, incubated for 48 h, and then treated with 0.5 mM $H_2O_2$ for 15 min. After incuba-

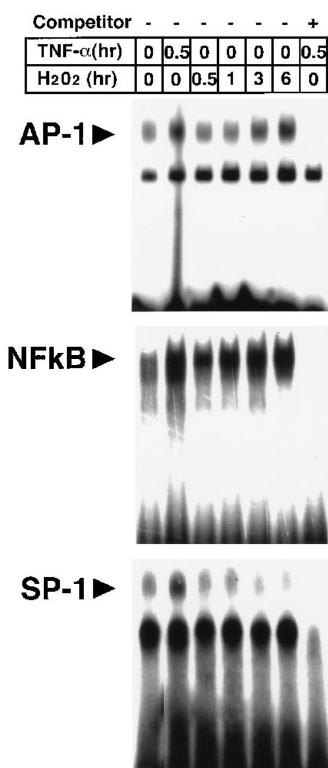



FIG. 3. Effect of TNF-α and $H_2O_2$ on nuclear factor binding to the AP-1, NF-κB, and Sp1 consensus fragments. Endothelial cells were incubated in serum-free medium 199 for 24 h and then exposed to TNF-α (100 U/ml) or $H_2O_2$ (0.5 mM) for 15 min. Endothelial cells treated with TNF-α or $H_2O_2$ were further incubated in drug-free medium for 15 min (shown as 0.5 h), 45 min (1 h), 2 h 45 min (3 h), and 5 h 45 min (6 h). The cells were harvested, and nuclear extracts were prepared. The extracts were incubated with $^{32}$P-labeled oligonucleotides of AP-1, NF-κB, and Sp1 and were resolved by gel electrophoresis. A 100-fold excess of the unlabeled oligonucleotides was added for the competition. The arrows indicate the retarded DNA-protein complexes of AP-1, NF-κB, and Sp1.

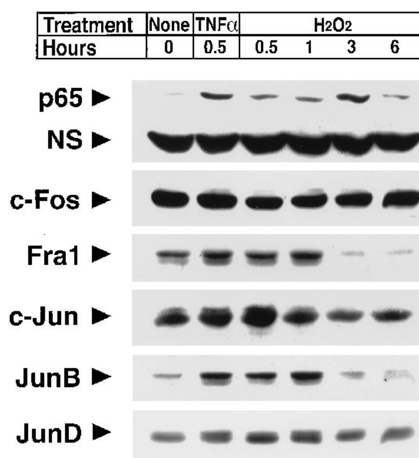



FIG. 5. Western blot analysis of NF-κB (p65), Fos family proteins, and Jun family proteins in nuclear extracts. Endothelial cells were incubated in serum-free medium 199 for 24 h, and TNF-α (100 U/ml) or $H_2O_2$ (0.5 mM) was added to the medium. After the indicated incubation period, cells were harvested and nuclear extracts were prepared. The lysates (25 μg) were fractionated by SDS–10% PAGE. NF-κB (p65), c-Fos, Fra-1, c-Jun, JunB, and JunD were detected by immunoblotting with polyclonal anti-NF-κB (p65), anti-c-Fos, and anti-c-Jun. NS, nonspecific.

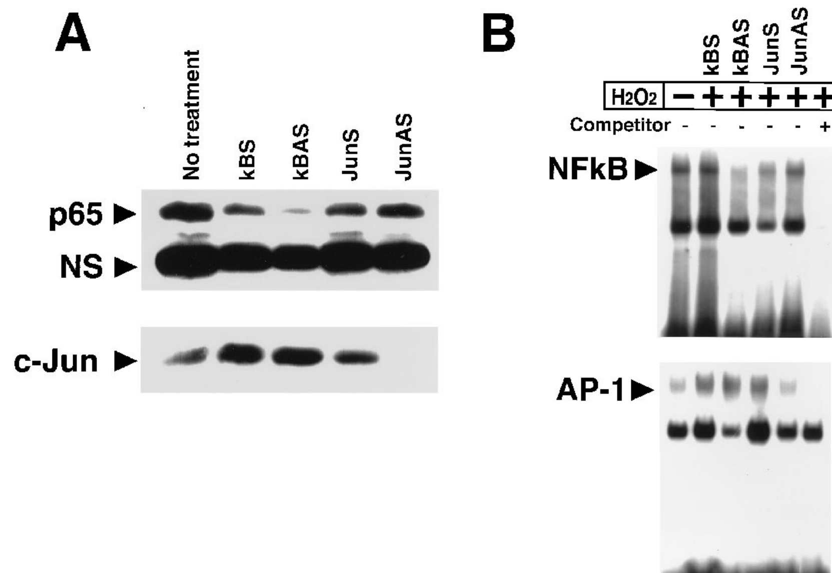
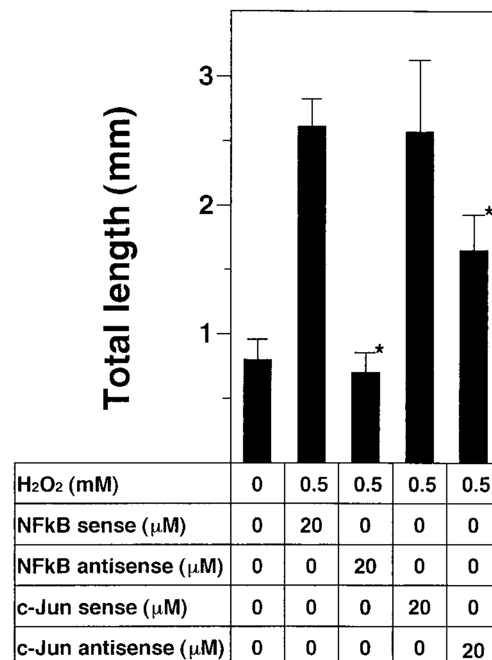



FIG. 6. Effect of antisense oligonucleotides of NF-κB and c-Jun. Endothelial cells were incubated in medium 199 containing 10% FBS with 20 μM sense or antisense NF-κB or c-Jun oligonucleotides for 48 h. (A) Cellular levels of p65 (NF-κB) and c-Jun. The whole-cell lysate was fractionated by SDS–10% PAGE. NF-κB (p65) and c-Jun were detected by immunoblotting with polyclonal anti-NF-κB (p65) and anti-c-Jun. NS, nonspecific. (B) Binding activities of NF-κB and AP-1. After treatment with 0.5 mM $H_2O_2$ for 15 min, medium 199 containing 10% FBS was replaced with serum-free medium 199. After an additional 6 h of incubation, the cells were harvested and nuclear extracts were prepared. The extracts were incubated with $^{32}$P-labeled oligonucleotides of AP-1 or NF-κB and were resolved by gel electrophoresis. A 100-fold excess of the unlabeled oligonucleotides was added for the competition. The arrows indicate the retarded DNA-protein complexes of AP-1 and NF-κB.

tion for 3 days, the $H_2O_2$-induced formation of tube-like structures was observed. Tubular morphogenesis was quantified by measuring the total tube length developed in the collagen gel. Sense oligonucleotides of NF-κB or c-Jun did not inhibit tube formation. Pretreatment with the antisense oligonucleotides of NF-κB almost completely abrogated $H_2O_2$-induced tube formation (Fig. 1E and 7). The antisense oligonucleotides of c-Jun partially inhibited tube formation. NF-κB, rather than AP-1, appeared to be directly involved in $H_2O_2$-dependent tubular morphogenesis in human microvascular endothelial cells.

**Induction of IL-8 mRNA by $H_2O_2$ stress.** The increased expression of angiogenic factors TGF-α, VEGF, IL-8, bFGF, hepatocyte growth factor, and TNF-α after $H_2O_2$ treatment was examined by Northern blot analysis. As a control, we examined the effects of treatment with TGF-α and IL-8. Treatment with 0.5 mM $H_2O_2$ for 15 min enhanced mRNA levels of VEGF 4- and 8-fold and enhanced mRNA levels of IL-8 28- and 23-fold at 3 and 6 h, respectively (Fig. 8). No apparent enhancement of mRNA levels of TGF-α (Fig. 8), bFGF, hepatocyte growth factor, or TNF-α (data not shown) was observed when cells were treated with $H_2O_2$. Treatment with TGF-α did not enhance TGF-α mRNA levels but increased mRNA levels of VEGF and IL-8 fivefold and fourfold, respectively. Treatment with IL-8 did not enhance the cellular mRNA levels of TGF-α and IL-8, and VEGF mRNA was increased only twofold over the initial level (Fig. 8). Of angiogenic factors, IL-8 had the most dramatic $H_2O_2$-induced enhancement of expression.

**Inhibition of $H_2O_2$-induced tubular morphogenesis by IL-8 antibody.** The development of tube-like structures (Fig. 9A) was increased in a dose-dependent manner in the presence of IL-8 at 1 to 100 ng/ml. Total tube length was estimated to be 0.41 ± 0.09 mm in the absence of growth factors, 2.14 ± 0.35 in the presence of 30 ng of TGF-α per ml, and 0.71 ± 0.14, 0.92 ± 0.19, 2.06 ± 0.26, and 2.28 ± 0.21 mm in the presence




| H2O2 (mM) | 0 | 0.5 | 0.5 | 0.5 | 0.5 |
|---|---|---|---|---|---|
| NFkB sense (μM) | 0 | 20 | 0 | 0 | 0 |
| NFkB antisense (μM) | 0 | 0 | 20 | 0 | 0 |
| c-Jun sense (μM) | 0 | 0 | 0 | 20 | 0 |
| c-Jun antisense (μM) | 0 | 0 | 0 | 0 | 20 |

FIG. 7. Inhibition of $H_2O_2$-induced tube formation by antisense oligonucleotides of NF-κB and c-Jun. Endothelial cells were plated onto collagen gels in medium 199 containing 10% FBS and were grown until nearly confluent. Sense or antisense NF-κB or c-Jun oligonucleotides (20 μM) were added to the medium, and the mixture was incubated for 48 h. After 15 min of $H_2O_2$ treatment, the medium was replaced with medium 199 containing 1% FBS for 3 days of incubation. Tube formation was then quantified. *, statistically significant difference ($P < 0.01$) compared with the value obtained by using the corresponding sense oligonucleotide.

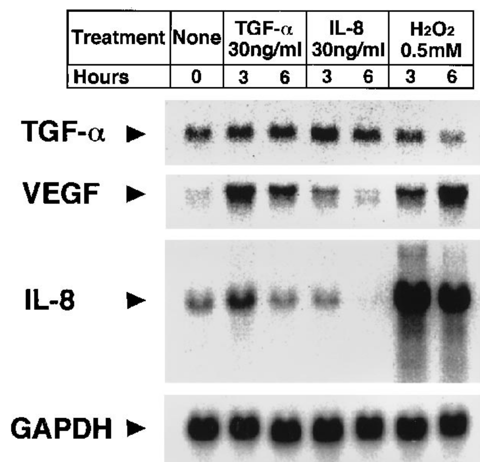



FIG. 8. Effect of $H_2O_2$ on the expression of TGF-α, VEGF, and IL-8 mRNA. Endothelial cells were incubated in serum-free medium 199 for 24 h, exposed to TGF-α (30 ng/ml) and IL-8 (30 ng/ml) for 3 and 6 h, respectively, and to $H_2O_2$ (0.5 mM) for 15 min, and then incubated in $H_2O_2$-free media for 2 h 45 min (shown as 3 h) and 5 h 45 min (6 h). At indicated times, cells were collected and mRNAs were hybridized with $^{32}$P-labeled TGF-α, VEGF, IL-8, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. The radioactivity of the corresponding area was measured with a BAS2000 bioimage analyzer. The cellular levels of TGF-α, VEGF, and IL-8 mRNA were normalized by the GAPDH mRNA level.

of 1, 10, 30, and 100 ng of IL-8 per ml, respectively. The tube formation of the microvascular endothelial cells was induced by IL-8 as well as TGF-α.

We examined whether $H_2O_2$-induced formation of tube-like structures could be mediated through paracrine control of IL-8 or other factors. Vascular endothelial cells were exposed to 0.5 mM $H_2O_2$ for 15 min, and then the medium was replaced with medium 199 containing 1% FBS with or without antibodies against IL-8 or VEGF. Coadministration of the anti-IL-8 antibody caused an almost complete abrogation of the $H_2O_2$-dependent formation (Fig. 1F and 9B). However, coadministration of the anti-VEGF antibody caused only a slight inhibition of $H_2O_2$-dependent tube formation.

**Inhibition of IL-8 production by antisense oligonucleotides of NF-κB.** Tubular morphogenesis enhanced by $H_2O_2$ was blocked by NF-κB antisense oligonucleotides (Fig. 6 and 7). To determine whether NF-κB was specifically involved in IL-8 production, cellular IL-8 levels were determined in the medium when the endothelial cells, treated with NF-κB sense or antisense oligonucleotides, were further exposed to $H_2O_2$. When the endothelial cells became confluent, 20 μM NF-κB or c-Jun sense or antisense oligonucleotides was added to the medium and the mixture was incubated for 48 h. Then the cells were exposed to 0.5 mM $H_2O_2$ for 15 min, the medium was replaced with 0.5 ml of serum-free medium 199, and the mixture was incubated for 6 h. The medium was collected and subjected to ELISA. IL-8 production was increased 10-fold over the control by $H_2O_2$ treatment (Fig. 10). NF-κB antisense oligonucleotides greatly interfered with $H_2O_2$-dependent IL-8 production, while the inhibitory effect of c-Jun antisense oligonucleotides was much lower.

## DISCUSSION

Shatos et al. (56) have demonstrated that brief exposure of human umbilical endothelial cells to oxidative stress causes a profibrinolytic response consisting of increased tissue-type plasminogen and plasminogen activator inhibitor-1 activities. Our study demonstrates that brief exposure enhances tubular morphogenesis of human microvascular endothelial cells. $H_2O_2$ stimulates the formation of tube-like structures of microvascular endothelial cells at rates comparable to those of EGF/TGF-α (44, 47) and VEGF (62). The formation of tube-like structures was also demonstrated in type I collagen gels with bovine aortic endothelial cells and human umbilical vascular endothelial cells exposed to $H_2O_2$ (unpublished data). These findings suggest that tubular morphogenesis of vascular endothelial cells caused by oxidative stress is ubiquitous. Our ob-

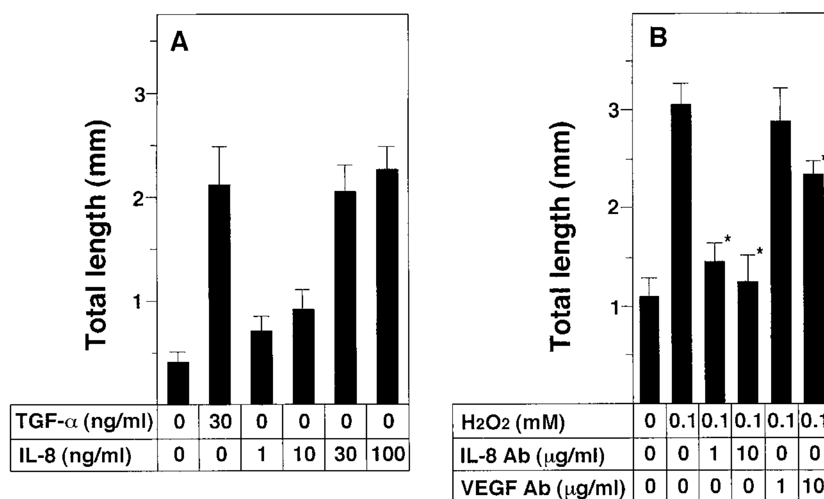



FIG. 9. (A) Development of tube-like structures in vascular endothelial cells in the presence of TGF-α and IL-8. Endothelial cells were plated on collagen gel and incubated in medium 199 containing 10% FBS. After the cells became confluent, the medium was replaced with medium 199 containing 1% FBS with the indicated doses of TGF-α and IL-8. (B) Inhibition of tube formation by antibodies against IL-8 or VEGF. The cells were exposed to 0.5 mM $H_2O_2$ for 15 min, and the medium was replaced by medium 199 containing 1% FBS with or without antibodies (Ab) against IL-8 or VEGF. The cells were incubated for 3 days, and phase-contrast microscopic pictures were recorded with a still video camera recorder. The total length of the tube-like structures was determined with an image analyzer. Eight random fields were measured, and the total length per field was calculated. Columns are means ± standard deviations (error bars) of triplicate experiments. *, statistically significant difference ($P < 0.01$) compared with the value obtained in the absence of antibodies.

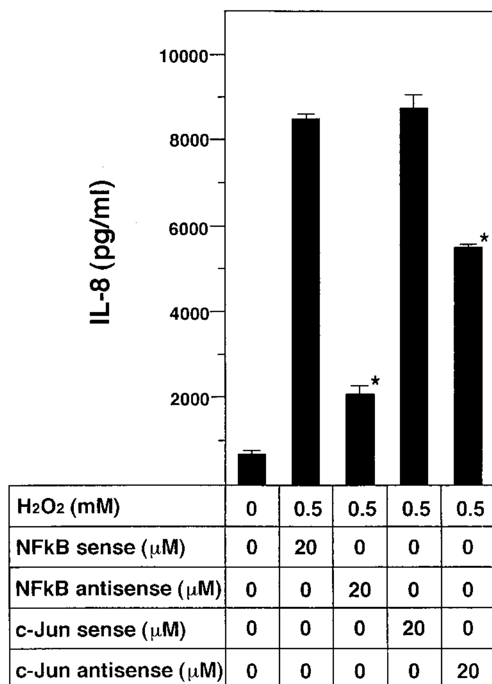



FIG. 10. Inhibition of IL-8 production by antisense oligonucleotides of NF-κB and c-Jun. Endothelial cells were plated onto the 24-well plastic plate in medium 199 containing 10% FBS. After the cells were confluent, 20 μM sense or antisense NF-κB or c-Jun oligonucleotide was added to the medium and incubated for 48 h. Then the cells were treated with 0.5 mM $H_2O_2$ for 15 min, and the medium was replaced with 0.5 ml of serum-free medium 199. After 6 h of incubation, the conditioned medium was collected and the IL-8 concentration was determined by ELISA. *, statistically significant difference ($P < 0.01$) compared with the value obtained by using the corresponding oligonucleotide.

servations are relevant to angiogenesis caused by inflammation, sepsis, and other pathologic conditions.

Oxygen radicals often use $H_2O_2$ as a second messenger to activate transcription factors. Several transcription factors—NF-κB (54, 55), AP-1 (2), and serum response factor/T-cell factor (35)—are involved in oxidative stress by $H_2O_2$ (48). The $H_2O_2$-induced angiogenesis in our model system was almost completely blocked by the coadministration of NF-κB antisense oligonucleotides, and was partially blocked by c-Jun antisense nucleotides. $H_2O_2$ stimulated the DNA-binding activities and cellular levels of NF-κB and AP-1, but not the DNA-binding activity of Sp1, in endothelial cells. The transcription factor NF-κB is a proto-oncogene member of the c-Rel family (19). NF-κB antisense oligonucleotides abrogate cell adhesion and tumorigenicity, resulting in tumor regression in the mouse model (18, 49, 58). It is unknown whether tumor angiogenesis is modulated by the administration of antisense oligonucleotides. Since NF-κB is activated by diverse pathogenic or pathogen-induced stimuli such as lipopolysaccharides, human immunodeficiency virus type 1, hepatitis B virus, radiation, and inflammatory cytokines (TNF and IL-1) (48), angiogenesis appearing in pathologic conditions might be mediated through NF-κB. Asakuno et al. (3) have reported that rat cells overexpressing c-Fos dramatically increase their survival and angiogenesis when transplanted in the rat brain. Although expression of AP-1 (Fos and Jun) was enhanced in response to $H_2O_2$, c-Jun appeared to be only partly involved in $H_2O_2$-dependent angiogenesis in the model system.

IL-8 is a potent neutrophil chemoattractant that induces cell migration of human umbilical endothelial cells and angiogenesis in rat cornea (25). IL-8 is implicated in angiogenesis in chronic inflammatory diseases such as rheumatoid arthritis and psoriasis (25, 43). Our study suggests a possible underlying mechanism for the involvement of NF-κB in $H_2O_2$-induced angiogenesis, on the basis of the following results. (i) $H_2O_2$ enhances the production of IL-8 in culture medium for endothelial cells to a level similar to that which induces angiogenesis. (ii) The exogenous addition of IL-8 induced the formation of tube-like structures at levels similar to those induced by the angiogenic factors EGF/TGF-α and TNF-α (34). (iii) $H_2O_2$ treatment-dependent tubular morphogenesis in vascular endothelial cells was completely abrogated by coadministration of IL-8 antibody. (iv) IL-8 production in endothelial cells could be blocked when the cells were treated with NF-κB antisense oligonucleotides. These findings support the theory that oxidative stress induces angiogenesis in the in vitro model system by IL-8 autocrine control, possibly through activation of NF-κB and partly through AP-1. IL-8 production was enhanced more than fivefold over the controls in human umbilical endothelial and human glioma cells (unpublished data). Oxidative stress by $H_2O_2$ enhances IL-8 production not only in vascular endothelial cells but also in other cell types, suggesting an involvement of paracrine control by those tumor cells producing IL-8 in angiogenesis. The IL-8 gene promoter contains binding sites for AP-1 (−126 to −120), NF-IL6 (−94 to −81), and NF-κB (−80 to −70) (32, 40, 46). Since the oxidative stress-induced activation of IL-8 was completely abrogated by NF-κB antisense oligonucleotides, $H_2O_2$-dependent IL-8 production might be due to NF-κB activation in human microvascular endothelial cells. However, it is unknown if NF-κB directly couples to the IL-8 gene in response to oxidative stress. Further studies with IL-8 promoter deletions need to be performed.

$H_2O_2$ enhances the expression of IL-8 and VEGF in vascular endothelial cells. However, $H_2O_2$ treatment did not enhance the expression of TGF-α, a potent angiogenic factor in microvascular endothelial cells (44, 47), or that of another potent angiogenic factor, bFGF (data not shown). VEGF, another potent angiogenic factor (8, 11), appears to be involved in brain tumor angiogenesis in both an animal model (23, 27) and patients (52). Our in vitro angiogenesis model, a human glioma cell line, induces tubular morphogenesis in microvascular endothelial cells through the paracrine control of VEGF/bFGF. Another cell line induces tubular morphogenesis through IL-8 (62). Expression of the VEGF gene is enhanced under hypoxic stress in human glioma cells (57) and in retinas in the mouse retinopathy model (50). Although $H_2O_2$ stimulates VEGF production in endothelial cells, coadministration of anti-VEGF antibody did not abrogate the oxidative stress-induced formation of tube-like structures; however, the anti-IL-8 antibody demonstrated complete inhibition. We propose that VEGF involvement in $H_2O_2$-dependent angiogenesis is unlikely.

TNF-α activates NF-κB (35, 48) and AP-1 (2, 35). In human microvascular endothelial cells, TNF-α promotes the DNA binding of NF-κB, AP-1, and Sp1 (15, 21) and the production of IL-6 and bFGF (45). Treatment of microvascular endothelial cells or glioma cells with TNF-α enhances the expression of Sp1 and VEGF, suggesting involvement of Sp1 in VEGF gene expression (51a). In our assay, brief exposure to TNF-α caused the induction of tubular morphogenesis. Coadministration of each NF-κB and c-Jun antisense oligonucleotide resulted in partial inhibition of TNF-α-induced angiogenesis (unpublished data), suggesting that other transcription factors besides NF-κB and c-Jun are involved in oxidative stress-induced angiogenesis. Our study demonstrated that long exposure (more than 6 h) to 100 U of TNF-α per ml inhibits plasminogen

activator gene expression, cell migration, and tubular morphogenesis in collagen gels and induces dramatic changes in cellular β-actin levels and the morphology of microvascular endothelial cells (26, 34). These findings indicate that prolonged treatment with this cytokine promotes the antiangiogenesis pathway in vascular endothelial cells. Human microvascular endothelial cells control dual pathways in response to TNF-α: angiogenesis promotion and inhibition. Early response to TNF-α, including expression of NF-κB or AP-1, might positively regulate angiogenesis. The later response to the same cytokine causes destabilization of β-actin mRNA and negative regulation of the angiogenesis process. Further investigation is required to understand the molecular mechanism for this regulation of angiogenesis by TNF-α.

In conclusion, ROIs are possible angiogenesis inducers that are mediated by the activation of the transcription factor NF-κB and possibly by the autocrine and/or paracrine control of IL-8. The use of the antibody against IL-8 or of NF-κB antisense oligonucleotides could play a valuable role in the therapy of angiogenesis-related diseases, such as diabetic retinopathy, tumors, rheumatoid arthritis, and psoriasis.

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