Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine that induces a broad spectrum of responses including angiogenesis. Angiogenesis promoted by TNF-α is mediated, at least in part, by ephrin A1, a member of the ligand family for Eph receptor tyrosine kinases. Although TNF-α induces ephrin A1 expression in endothelial cells, the signaling pathways mediating ephrin A1 induction remain unknown. In this study, we investigated the signaling mechanisms of TNF-α-dependent induction of ephrin A1 in endothelial cells. Both TNFR1 and TNFR2 appear to be involved in regulating ephrin A1 expression in endothelial cells, because neutralizing antibodies to either TNFR1 or TNFR2 inhibiting ephrin A1 expression. Inhibition of nuclear factor-κB (NF-κB) activation by a trans-dominant inhibitory isoform of mutant IκBα did not affect ephrin A1 induction, suggesting that NF-κB proteins are not major regulators of ephrin A1 expression. In contrast, ephrin A1 induction was blocked by inhibition of p38 mitogen-activated protein kinase (MAPK) or SAPK/JNK, but not p42/44 MAPK, using either selective chemical inhibitors or dominant-negative forms of p38 MAPK or TNF receptor-associated factor 2. These findings indicate that TNF-α-induced ephrin A1 expression is mediated through JNK and p38 MAPK signaling pathways. Taken together, the results of our study demonstrate that induction of ephrin A1 in endothelial cells by TNF-α is mediated through both p38 MAPK and SAPK/JNK, but not p42/44 MAPK or NF-κB, pathways.

Angiogenesis, the formation of new blood vessels, is a multistep process that includes endothelial cell proliferation, migration, capillary tube assembly, and recruitment of perivascular support cells to form mature and functional vessels (1, 2). This process is not only critical for embryogenesis and the normal function of the female reproductive tract but also plays an essential role under many pathological conditions such as wound healing, tumor growth and metastasis, and rheumatoid arthritis. Over the past decade, tremendous progress has been made in dissecting the molecular mechanisms underlying this important biological process. Receptor tyrosine kinases have emerged as critical molecules in regulating many aspects of angiogenesis (reviewed in Refs. 3 and 4). At least three families of receptor tyrosine kinases have been implicated in angiogenesis: the VEGF family, the angiopoietin/Tie2 family, and the ephrin/Eph family. VEGFs are vascular endothelial cell growth factors that promote endothelial cell proliferation, migration, and vessel assembly. Members of the VEGF receptor family are crucial to de novo blood vessel formation during embryonic development and mediate angiogenesis in a number of diseases including inflammation and cancer. Members of the angiopoietin/Tie2 family of receptor tyrosine kinases function in blood vessel remodeling, maturation, and stabilization. A third family, the Eph family, has recently been shown to significantly regulate angiogenesis.

The Eph family comprises the largest subfamily of receptor tyrosine kinases, including at least 14 receptors and 8 ligands. The binding of Eph ligands to their receptors is governed by cell-cell contact, because the Eph ligands, known as ephrins, are anchored to the cell surface (5, 6). Ephrins can be further divided into two groups, ephrin A and ephrin B subclasses, according to how they are anchored to the cell membrane (7–9). The ephrin A ligands are membrane-bound through glycosylphosphatidylinositol linkage, whereas ephrin B ligands are anchored to the membrane through transmembrane domains. The ephrin A subclass exhibits rather general and promiscuous binding to the EphA receptors, and the ephrin B subclass has a general binding preference for the receptors of the B subclass. Another unique feature of subclass B is that EphB receptors can activate ephrin B ligands through phosphorylation of their cytoplasmic domains (7, 8). Such bidirectional signaling may also exist between ephrin A ligands and Eph receptors, as documented in the case of ephrin A5 (9).

Functional evidence for involvement of Eph family receptor tyrosine kinases in angiogenesis came from both in vitro studies and gene knockout experiments. Targeted gene disruptions of ephrin B2 ligand and EphB4 receptor in mice revealed that the expression of ephrin B2 was restricted to arterial endothelium, whereas EphB4 was expressed in the venous endothelium during early vascular development. Embryos lacking ephrin B2 or EphB4 displayed severe defects in vascular remodeling in both arterial and venous domains (10, 11). Furthermore, EphB2 and B3 double knockout mice exhibited sim-
ilar vascular phenotypes (12). Another ligand-receptor pair, ephrin A1 and EphA2, has also been implicated in embryonic and adult angiogenesis. During early embryogenesis, ephrin A1 was expressed in developing vasculature (13), and ephrin A1 promoted angiogenesis in cornea in adult animals (14). Although ephrins generally do not induce endothelial cell proliferation, ephrin A1 could promote human umbilical vein endothelial cell (HUVEC) assembly into a capillary-like structure in Matrigel assays (15). Expression of ephrin A1 in HUVEC is regulated by the multifunctional proinflammatory cytokine TNF-α (16). Neutralizing antibodies against ephrin A1 inhibited TNF-α-induced angiogenesis in rabbit cornea assays (14), suggesting that ephrin A1 was a key mediator of TNF-α-induced angiogenesis.

TNF-α is a multifunctional cytokine that induces a broad spectrum of responses including angiogenesis. It is thought that TNF-α promotes angiogenesis through its ability to up-regulate the expression of various angiogenic factors. Multiple signaling pathways have been connected to TNF-α-induced gene induction. Two forms of TNF receptor have been identified, TNFR1 and TNFR2. A current model postulates that TNF binding triggers trimerization of TNFRs (TNFR1/2) and recruitment of adaptor proteins. The death domain protein TNFR-associated death domain protein binds to TNFR1 and serves as a platform for further recruitment of receptor-interacting protein, Fas-associated death domain, and TRAF2. Whereas Fas-associated death domain signals to the apoptotic protease cascade, receptor-interacting protein mediates p38 MAPK and NF-κB activation, and TRAF2 mediates JNK activation. Activation of TNF-κB, p38 MAPK, and JNK can, in turn, regulate a variety of cellular processes, including TNF-α-dependent gene induction (17, 18). To investigate how ephrin A1 is regulated by TNF-α, we investigated the signaling pathways involved in TNF-α-dependent up-regulation of ephrin A1 in endothelial cells. Here we report that, in contrast to the TNF-induced expression of other angiogenic factors, NF-κB is not a prime regulator of ephrin A1 expression. Rather, activation of p38 MAPK and SAPK/JNK pathways by TNF-α leads to ephrin A1 expression in endothelium.

**EXPERIMENTAL PROCEDURES**

**Endothelial Cell Culture—HUVEC were purchased from Clonetics and maintained in 10-mm dishes in endothelial cell basal medium with growth factors (Clonetics). Cells were subcultured up to passage 5. Human endothelial cell line ECV304 was maintained in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum in 10 cm dishes as described (19). Endothelial cells were cultured to 80% confluence, starved for 16 h in medium deficient in growth factors, preincubated with cycloheximide (CHX) at 10 μg/ml for 30 min, and then stimulated with recombinant human TNF-α (R & D Systems) at 20 ng/ml (unless otherwise indicated in figure legends). ECV cells were starved in serum-free Dulbecco’s modified Eagle’s medium overnight prior to various treatments as described below.

To inhibit NF-κB activity, HUVEC were incubated with adenoviruses overexpressing a dominant negative form of mutant IκB-α protein (AdIκBα) or with control virus expressing β-galactosidase (Ad-βgal) (generously provided by Dr. L. Kerr) at 10^9 plaque-forming units/ml for 24–40 h, prior to the addition of CHX and TNF-α. For chemical inhibition studies, endothelial cells were preincubated with the following chemical inhibitors prior to CHX and TNF-α stimulation: p38/44 MAPK-specific inhibitor PD98059 (New England Biolabs) at 50 μM for 1 h, p38 MAPK-specific inhibitor SB20358 (Sigma) at 2.5 μM, and JNK inhibitor DMAP (Sigma) at 1 μM for 15 min. To inhibit p38 MAPK and SAPK/JNK, cells were infected with adenoviruses overexpressing a dominant form of mutant p38 MAPK protein (Ad-p38MAPK.DN) or a trans-dominant inhibitory isoform of mutant TRAF2 protein (Ad-TRAF2.DN) for 24–40 h at 10^9 plaque-forming units/ml prior to stimulation with TNF-α (see below). To inhibit TNF-α receptor function, neutralizing antibodies (R & D Systems) to TNFR1 or TNFR2 were added to endothelial cell cultures at 5 μg/ml for 1 h prior to stimulation with TNF-α.

**Northern Blot Analysis—**RNA from stimulated HUVEC and ECV304 cells were isolated using Trizol reagent and chloroform (Life Technologies, Inc.). 10 μg of total RNA were resolved on a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech), and hybridized for 16–24 h to a 32P-labeled ephrin A1 cDNA probe. Membranes were washed once with 2× SSC at room temperature, followed by two stringent washes (1× SSC at 65 °C for 10 min and 0.5× SSC at 65 °C for 10 min), and exposed to Biomax film (Eastman Kodak Co.). The RNA blots were stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe as loading control. ECV304 RNA levels were quantified using the ImageQuant 1.62 software program.

**Whole Cell Extract Preparation and Western Blot Analysis—**HUVEC and ECV304 cells were lysed in SDS sample buffer, and cell extracts were cleared by sonication and centrifugation. 50 μg of protein from cell lysates were fractionated on a 12% SDS-polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane (ECL +, Amersham Pharmacia Biotech). Membranes were blocked for 1 h at room temperature with Tris-buffered saline containing 0.1% Tween 20 and 5% powdered nonfat milk and then incubated with antibodies against phosphorylated forms of JNK/SAPK, p38 MAPK, p42/44 MAPK, or ATF2 according to the manufacturer’s instructions (New England Biolabs). Immunoreactive proteins were detected with secondary antibodies (Santa Cruz Biotechnologies and Promega) conjugated to horseradish peroxidase using enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Construction of Adenoviruses—**Adenoviruses expressing dominant negative forms of mutant TRAF2 (Ad-TRAF2.DN) or p38 MAPK (Ad-p38MAPK.DN) were constructed according to the methods of Becker et al. (20). Briefly, TRAF2 cDNA truncated at the NH₂ terminus at codon 241 was generated by polymerase chain reaction using Pfu DNA polymerase (Stratagene) and subcloned into BamHI and HindIII sites of pcMV5 vector to create pCMVTRAF2(241–501). The pCMV5 vector containing a p38 MAPK cDNA containing Thr-180 → Ala and Tyr-182 → Phe substitutions was cloned from pCMV5 vector (a gift from Dr. R. Davies) into HindIII and XbaI sites of pAC-CMV to create pACCMVp38MAPK.DN. The plasmid constructs pAC-CMVMTRF2(241–501).DN or pAC-CMVp38MAPK.DN were cotransfected with pJM17 (a gift from Dr. L. Kerr) into the 293T cells packaging line for viral production. The resulting virus was further amplified in 293T cells and purified by a PD10 column (Centriplus).

**Electrophoretic Mobility Shift Assay—**HUVEC were infected with AdIκBα or Ad-βgal at 10^6 plaque-forming units/ml for 24–48 h, folowed by the addition of TNF-α for 2 h. Nuclear fractions were prepared by high salt extraction in the presence of protease inhibitors (tosylphenylalanine chloromethyle ketone and tolyssilane chloromethylene ketone at 100 μM, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin at 100 μg/ml). Gel mobility shift assays were performed by using a 32P-labeled oligonucleotide duplex derived from B enhancer sequences in the interleukin-2 receptor-α promoter region (5′-CAACGCCAGGGGAATCCCTCCTCTT-3′) (21). The DNA binding reaction mix containing 5 μg of nuclear extracts, 2 μg of double-stranded poly(dI-dC), 10% Nonidet P-40, 0.1% dithiothreitol, and 10 μg of bovine serum albumin were buffered in 10× HGE (200 mM HEPES, 50% glycerol, 1 mM EDTA). The resultant binding complex was resolved on a native 5% acrylamide gel and visualized by autoradiography (22).

**Kinase Assay—**In vitro kinase assay was performed as described (17). Briefly, cell extracts were prepared in Triton lysis buffer (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 2 mM EGTA, 1 mM Na3VO4, 25 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium pyrophosphate, 15% (v/v) glycerol, and 1% (v/v) Triton X-100). 900 μg of protein were immunoprecipitated with 0.8 μg/ml anti-p38 MAPK antibodies (Santa Cruz Biotechnology). For kinase reaction, 1.5 μg of the glutathione S-transferase-c-Jun (New England Biolabs) and 20 μg ATP were incubated with the immunoprecipitated p38 MAPK in kinase reaction buffer for 20 min at 30 °C. The reaction mix was resolved via 15% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

**RESULTS**

**Kinetics and Concentration Dependence of TNF-α-induced Ephrin A1 Expression in Endothelial Cells—**To determine the kinetics of ephrin A1 regulation, HUVEC were stimulated with 20 ng/ml TNF-α for 15 min to 16 h. As shown in Fig. 1A, unstimulated cells did not express a significant amount of ephrin A1 mRNA. However, upon treatment with TNF-α, induction was seen as early as 15 min after stimulation, Maxim
mum induction was reached in 2 h, and mRNA levels began a steady decline thereafter. TNF-α was also active on the human endothelial cell line ECV304, with a time course similar to that of HUVEC. The induction of ephrin A1 by TNF-α was dose-dependent, with maximum induction at 20–40 ng/ml (Fig. 1B).

**Induction of Ephrin A1 Expression by TNF-α Is Mediated through Both TNFR1 and TNFR2**—TNF-α induces the expression of many genes through two different TNF receptors. TNFR1 (p55) and TNFR2 (p75) bind to TNF-α with different affinities and have been shown to activate distinct, as well as overlapping, pathways (23, 24). To determine which receptor is involved in regulation of ephrin A1 expression, endothelial cells were treated with neutralizing monoclonal antibodies specifically inhibitory isoform of IκBα (AdmIκBα). As shown in Fig. 2, neutralizing antibodies against either TNFR1 or TNFR2 (25). As shown in Fig. 2, neutralizing antibodies against either TNFR1 or TNFR2 inhibited ephrin A1 expression. These results indicate that both TNF-α receptors are involved in regulating ephrin A1 expression.

**Inhibition of NF-κB Activation Does Not Affect Ephrin A1 Expression**—The NF-κB family of transcription factors is one of the main mediators of the intracellular functions of TNF-α. In the quiescent state, NF-κB proteins are sequestered in the cytoplasm by binding to a family of inhibitory proteins including IκBα. During TNF-α-induced cell activation, IκBα undergoes signal-induced phosphorylation and ubiquitin-proteasome-mediated degradation, permitting the nuclear import of NF-κB to activate transcription. Several TNF-α-induced angiogenic molecules such as VEGF, VEGFβ2 (βk-1), E-selectin, and ICAM-1 (26–29) have been shown to be regulated through an NF-κB-dependent mechanism. Because ephrin A1 is also a TNF-α-induced angiogenic factor, we determined whether NF-κB proteins are also involved in mediating ephrin A1 expression.

To inhibit the NF-κB signaling pathway, HUVEC were infected with an adenovirus overexpressing a trans-dominant inhibitory isoform of IκBα (AdmIκBα). This mutant IκBα contains serine to alanine point mutations at positions 36 and 40 that confer resistance to signal-dependent phosphorylation and degradation, thus functioning as a constitutive repressor of multiple NF-κB proteins (30, 31). Gel mobility shift analyses were performed with nuclear extracts from TNF-α-stimulated cells infected with either AdmIκBα or a control virus expressing β-galactosidase (Ad-βgal). As shown in Fig. 3A, TNF-α stimulated high levels of nuclear NF-κB activity in uninfected cells or cells infected with Ad-βgal, whereas the nuclear levels of NF-κB were undetectable in cells infected with AdmIκBα. These results suggest that overexpression of dominant-negative IκBα inhibited NF-κB activation in endothelial cells. However, although AdmIκBα-treated cells no longer demonstrated detectable induction of NF-κB by TNF-α, the induction of ephrin A1 by TNF-α was not affected (Fig. 3B), indicating that NF-κB is not a prime regulator of TNF-α-induced ephrin A1 expression in endothelial cells.

**Inhibition of p38 MAPK and SAPK/JNK Activation Blocks TNF-α-induced Ephrin A1 Expression**—In addition to the NF-κB pathway, TNF-α binding to its receptors activates multiple pathways that culminate in altered activity of transcription factors. One of the central mediators that propagate signals from the cell membrane to the nucleus is the MAP kinase...
superfamily. At least three different subtypes of MAP kinases are known: p42/44 MAPK, p38 MAPK, and SAPK/JNK. To further investigate whether the MAP kinases mediate TNF-α-induced ephrin A1 expression in endothelial cells, selective chemical inhibitors PD98059, SB20358, and DMAP were used to inhibit p42/44 MAPK, p38 MAPK, and SAPK/JNK, respectively. DMAP has been shown to inhibit phosphorylation and activation of JNK in endothelial cells (32). PD98059 has been shown to selectively inhibit phosphorylation of p42/44 MAPK but not p38 MAPK or SAPK/JNK at the concentration of 50 μM (33). In contrast, SB20358 does not inhibit the phosphorylation of p38 MAPK; rather, it binds to p38 MAPK and inhibits the ability of p38 MAPK to phosphorylate downstream target proteins (34).

Endothelial cells were preincubated with the selective MAPK inhibitors DMAP, SB20358, or PD98059 and stimulated with TNF-α, and ephrin A1 mRNA induction was analyzed by Northern blot analysis. Duplicate plates were treated under the same conditions, and cell extracts were prepared for Western blot analysis to determine the levels of MAPK inhibition. As shown in Fig. 4A, cells pretreated with either DMAP or SB20358 resulted in a significant reduction of ephrin A1 expression. The inhibition of ephrin A1 induction was also dose-dependent, because 7 μM SB20358 significantly suppressed the induction, and 100 μM DMAP completely blocked the induction (Fig. 4B). In contrast, ephrin A1 expression was not affected in the presence of the p42/44 MAPK inhibitor PD98059 (Fig. 4A). Western blot analysis (Fig. 4C) confirmed previous investigations showing that PD98059 selectively inhibits phosphorylation of p42/44 MAPK but does not affect p38 MAPK or SAPK/JNK. Although SB20358 does not prevent phosphorylation of p38 MAPK, it inhibits the ability of p38 MAPK to phosphorylate downstream effector ATF2. The specificity of DMAP was demonstrated by inhibition of phosphorylation of SAPK/JNK. Although DMAP had been shown to also inhibit p38 MAPK in mouse Sertoli cells (35), in the present experiments there was no detectable effect on p38 MAPK or p42/44 MAPK in endothelial cells at a concentration of 1 mM (Fig. 4C). Taken together, these data suggest that induction of ephrin A1 expression by TNF-α was mediated through p38 MAPK and SAPK/JNK pathways but was independent of p42/44 MAPK.

**Ephrin A1 Induction Is Mediated through p38 MAPK- and TRAF2-dependent Mechanisms**—Because chemical inhibition might affect other unknown downstream effectors of TNF receptors, we used an independent approach to address whether p38 MAPK and SAPK/JNK mediate ephrin A1 induction by TNF-α. Recombinant adenoviruses expressing dominant negative p38 MAPK (Ad-p38MAPK.DN) and dominant negative TRAF2 (Ad-TRAF2.DN) were generated and used for infection of HUVEC and ECV cells. Dominant negative p38 MAPK contains Thr-180→Ala and Tyr-182→Phe substitutions, resulting in resistance to activation-induced phosphorylation. TRAF2 is an adaptor protein that binds to the TNFR/TRADD complex and has been shown to specifically mediate JNK activation (17, 36). Dominant negative TRAF2 truncated at codon 241 at the NH2 terminus and was previously shown to inhibit JNK activity (36, 37). As shown in Fig. 5D, overexpression of dominant negative p38 MAPK significantly inhibited the ability of p38 MAPK to phosphorylate c-Jun, a downstream effector of p38 MAPK. Furthermore, expression of dominant negative TRAF2 did not affect the phosphorylation status of p38 MAPK but did inhibit JNK phosphorylation (Fig. 5C), suggesting that TRAF2 specifically mediated JNK activation but not p38 MAPK in endothelial cells. Consistent with our chemical inhibitor data, TNF-α-induced ephrin A1 expression was inhibited by both Ad-p38MAPK.DN and Ad-TRAF2.DN, whereas Ad-βgal did not affect ephrin A1 expression (Fig. 5, A and B). These results provide direct evidence of the involvement of p38 MAPK and SAPK/JNK pathways in TNF-α-induced ephrin A1 expression and further indicated that TRAF2 acted upstream of JNK to activate the JNK pathway.

**DISCUSSION**

TNF-α is a multifunctional cytokine that induces a broad spectrum of responses, including cell growth, apoptosis, induction of other cytokines, and angiogenesis. TNF-α apparently promotes angiogenesis through indirect effects, mediated by its regulation of angiogenic factors. In addition to inducing ephrin A1 expression, TNF-α up-regulates the expression of VEGF2 (flk-1), basic fibroblast growth factor, platelet activating factor, tissue factors, E-selectin, and ICAM-1 (26, 35, 38, 39). However, signaling pathways mediates TNF-α-dependent induction in endothelial cells have not been thoroughly investigated. In this report we studied the signaling mechanisms that mediate TNF-α-induced ephrin A1 expression in endothelial cells. We showed that (1) both TNFR1 and TNFR2 are involved in regulating ephrin A1 expression in endothelial cells, (2) the induction of ephrin A1 is TRAF2-dependent, (3) activation of p38 MAPK or SAPK/JNK, but not p42/44 MAPK, signaling pathways lead to ephrin A1 induction, and (4) activation of NF-κB is not required for TNF-α-induced ephrin A1 expression.

The many functions of TNF are mediated by two cell surface receptors, TNFR1 and TNFR2. TNF binding induces receptor aggregation, resulting in the recruitment of different types of intracellular signal transducers to the TNFR complexes. One of these signaling transducers is TRAF2, which interacts with the cytoplasmic tails of both TNFRs and serves as an adaptor...
protein to recruit downstream signal transducers. Recent gene disruption and dominant negative transgenic studies demonstrate that TRAF2 is required for JNK activation but not NF-κB activation (17, 18) in lymphocytes and embryonic fibroblasts. However, it is not clear what downstream kinase cascades are mediated by TRAF2 in endothelial cells. Here we

![FIG. 4. Chemical inhibition of p38 MAPK and SAPK/JNK activation blocks TNF-α-induced ephrin A1 expression.](image)

A. ECV304 cells were pre-incubated with selective MAPK inhibitors (1 mM DMAP, 50 μM PD98059, or 5 μM SB20358) prior to stimulation with TNF-α. 10 μg of total RNA were isolated and subjected to Northern analysis for ephrin A1 expression, as described in the legend to Fig. 1A. B. ECV304 cells were pre-incubated with increasing doses of SB20358 or DMAP and stimulated with TNF-α, and RNAs were subjected to Northern blot analysis. C, whole cell extracts were pre-incubated from ECV304 cells pre-incubated with chemical inhibitors and stimulated with TNF-α. 50 μg of protein/lane were resolved via 12% SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and blotted with antibodies to phosphorylated forms of p38 MAPK, SAPK/JNK, p42/44 MAPK, or ATF2. The membranes were then stripped and rebotted with antibodies to the unphosphorylated forms of proteins for loading controls. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; unstim, unstimulated.

![FIG. 5. Dominant negative forms of TRAF2 and p38 MAPK inhibit TNF-dependent ephrin A1 induction.](image)

A. HUVEC and ECV304 cells were infected with adenovirus overexpressing dominant negative TRAF2 (Ad-TRAF2.DN), dominant negative p38 MAPK (Ad-p38MAPK.DN), or Ad-β-gal for 24–40 h and stimulated with TNF-α. 10 μg of total RNA were subjected to Northern analysis for ephrin A1 expression. One of the three representative experiments is shown here. B, levels of ephrin A1 induction in triplicate experiments were quantified using the Scion Image 1.62 software program and calculated as the ephrin A1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ratio (*, p < 0.05 versus TNF-α positive control; Student’s t test). C, Western blot analysis showing that dominant negative TRAF2 inhibits the phosphorylation of JNK but not p38 MAPK. D, cell lysates prepared from endothelial cells unstimulated (−) or stimulated (+) with TNF (10 ng/ml) in the presence (+) or absence (−) of either Ad-p38MAPK.DN or Ad-β-gal were used to measure p38 MAPK activity by immunocomplex kinase assay with GST-c-Jun as a substrate.
show that as in lymphocytes and embryonic fibroblasts, TRAF2 mediates JNK activation in HUVEC and ECV cells. However, in contrast to published reports in 293 cells (36, 40), TRAF2 apparently does not mediate p38 MAPK activation in endothelial cells, because the phosphorylation of p38 MAPK was not affected by a dominant negative TRAF2. Thus, inhibition of ephrin A1 induction by dominant negative TRAF2 is mediated by the JNK pathway.

In endothelial cells, at least three different subtypes of MAP kinases are known to be activated by TNF-α: p42/p44 MAPK, p38 MAPK, and SAPK/JNK. p38 MAPK and SAPK/JNK have been shown to regulate expression of gene products involved in stress-related events (41). In particular, p38 MAPK activation mediates actin reorganization and cell migration in human endothelial cells. In contrast, p42/p44 MAPK has been shown to be involved in cell proliferation (42). Here we show that inhibition of p38 MAPK and SAPK/JNK, but not p42/44 MAPK, either by selective chemical inhibitors or dominant negative p38 MAPK or TRAF2 blocked ephrin A1 induction by TNF-α. Regulation of ephrin A1 expression through SAPK/JNK and p38 MAPK is consistent with the functional role of ephrin A1, which has been shown to induce cell migration and vessel assembly (14) but not cell proliferation.

NF-κB proteins are key proinflammatory transcription factors that mediate TNF-α-dependent gene induction events. NF-κB proteins have been implicated in the induction of several angiogenic molecules. For example, VEGF/F2 (flk-1/KDR) expression is induced by TNF-α, and this induction is mediated through NF-κB in combination with cAMP-response element-binding protein and histone acetylases (27). NF-κB is also involved in the regulation of E-selectin and VCAM-1; the soluble forms of the encoded proteins induce angiogenesis (28, 43). However, our gel mobility shift studies and Northern blot analysis showed that TNF-α-induced ephrin A1 expression is not mediated through an NF-κB-dependent mechanism. These results are consistent with our chemical inhibition studies in which curcumin inhibited NF-κB activation but did not affect ephrin A1 induction (data not shown). It is currently not known what transcription factors are required for ephrin A1 induction in endothelial cells, and the ephrin A1 promoter elements have not been identified. However, HoxB3, a TNF-α-inducible homeobox transcription factor that promotes capillary morphogenesis and angiogenesis, induces ephrin A1 expression in endothelial cells (44), suggesting that HoxB3 may mediate TNF-α-induced ephrin A1 expression. Because activation of p38 MAPK and SAPK/JNK leads to ephrin A1 induction, AP-1 or ATF2 transcription factors downstream of p38 MAPK and SAPK/JNK may also be involved in regulation of ephrin A1.

In summary, our data indicate that induction of ephrin A1 in endothelial cells by TNF-α is mediated through both p38 MAPK and SAPK/JNK (Fig. 6), but not p42/p44 MAPK or NF-κB, pathways. The TNF-α-mediated up-regulation of ephrin A1 is likely to play an important role in angiogenesis under physiological conditions and in pathological diseases such as tumor growth and metastasis, rheumatoid arthritis, and diabetic retinopathy. Thus characterizing the signaling mechanisms that regulate TNF-α-dependent ephrin A1 induction may elucidate new targets for therapeutic intervention.

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