**Angiogenesis, Metastasis, and the Cellular Microenvironment**

**TGF-β1 Induces Endothelial Cell Apoptosis by Shifting VEGF Activation of p38MAPK from the Prosurvival p38β to Proapoptotic p38α**

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**Abstract**

TGF-β1 and VEGF, both angiogenesis inducers, have opposing effects on vascular endothelial cells. TGF-β1 induces apoptosis; VEGF induces survival. We have previously shown that TGF-β1 induces endothelial cell expression of VEGF, which mediates TGF-β1 induction of apoptosis through activation of p38 mitogen-activated protein kinase (MAPK). Because VEGF activates p38 MAPK but protects the cells from apoptosis, this finding suggested that TGF-β1 converts p38 MAPK signaling from prosurvival to proapoptotic. Four isoforms of p38 MAPK — α, β, γ, and δ — have been identified. Therefore, we hypothesized that different p38 MAPK isoforms control endothelial cell apoptosis or survival, and that TGF-β1 directs VEGF activation of p38 MAPK from a prosurvival to a proapoptotic isoform. Here, we report that cultured endothelial cells express p38α, β, and γ. VEGF activates p38β, whereas TGF-β1 activates p38α. TGF-β1 treatment rapidly induces p38α activation and apoptosis. Subsequently, p38α activation is downregulated, p38β is activated, and the surviving cells become refractory to TGF-β1 induction of apoptosis and proliferate. Gene silencing of p38α blocks TGF-β1 induction of apoptosis, whereas downregulation of p38β or p38γ expression results in massive apoptosis. Thus, in endothelial cells p38α mediates apoptotic signaling, whereas p38β and p38γ transduce survival signaling. TGF-β1 activation of p38α is mediated by VEGF, which in the absence of TGF-β1 activates p38β. Therefore, these results show that TGF-β1 induces endothelial cell apoptosis by shifting VEGF signaling from the prosurvival p38β to the proapoptotic p38α. *Mol Cancer Res; 1–10.* © 2012 AACR.

**Introduction**

Angiogenesis, the formation of capillaries from preexisting blood vessel, is mediated by a variety of cytokines and growth factors with paracrine or autocrine modes of action. VEGF and TGF-β1, potent angiogenesis inducers, act on vascular endothelial cells through different mechanisms (1–3).

VEGF (VEGF A), the prototype member of a family of 4 growth factors (VEGF A–D), upregulates endothelial cell proliferation and migration and protects endothelial cells from apoptosis (1). VEGF exerts its activity through 2 tyrosine kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (flk-1). VEGFR-2 has been implicated in endothelial cell proliferation and survival, and VEGFR-1 in chemotaxis and vascular permeability (1, 4). Protein kinase B (Akt) and mitogen-activated protein kinases (MAPK) are components of the signaling mechanism activated by VEGFR-2 (5).

TGF-β1, the prototype member of a superfamily of 5 multifunctional growth factors, is a potent proliferation inhibitor for most cell types, and an important regulator of tissue morphogenesis (3, 6). TGF-β1 induces vessel formation in vitro and in vivo (7–11); however, it inhibits endothelial cell proliferation and migration (12) and downregulates VEGFR-2 expression (13, 14). Notably, TGF-β1 induces endothelial cell apoptosis (12, 15) by inhibiting expression of the antiapoptotic protein Bcl-2 (16) and activating p38 MAPK (p38 MAPK; ref. 17).

Thus, although both VEGF and TGF-β1 induce angiogenesis, they have opposing effects on endothelial cells. Remarkably, TGF-β1 is a potent inducer of apoptosis, whereas VEGF protects the cells from apoptosis. It has therefore been proposed that TGF-β1 induces angiogenesis in vivo through an indirect mechanism, by recruiting inflammatory cells that in turn secrete VEGF and/or other angiogenesis inducers. However, TGF-β1 induces endothelial cell expression of VEGF in vitro and in vivo (18–20) and, surprisingly, VEGF is required for induction of endothelial cell apoptosis through VEGFR-2 activation of p38 MAPK (19, 20).
p38MAPK, a class of MAPK activated by environmental stresses, growth factors and cytokines, controls cell functions including proliferation and apoptosis, differentiation, and senescence (21, 22). Four isoforms of p38MAPK—α, β, γ, and δ—have been identified in mammalian cells. Despite more than 60% sequence homology and more than 90% identity within their kinase domains, the p38MAPK isoforms show notable differences in tissue expression, upstream activators, and downstream effectors. p38α and p38β are ubiquitous; p38γ and p38δ are tissue specific (22). In addition, the p38 isoforms have been described in different cell compartments, in which they can have opposing effects on the same substrate (21). The specific function(s) of the individual isoforms in physiology and pathology are largely unknown. The genetic deficiency of p38α in mice results in embryonic lethality, with aberrant placental development and abnormal angiogenesis in the yolk sac and embryo (23). In contrast, disruption of the other isoforms generates no apparent phenotype (21). Numerous studies have implicated p38MAPK in induction of endothelial cell apoptosis by a variety of agents (24–27). However, the p38 isoforms involved have not been characterized.

Here, we report that in vascular endothelial cells, p38α mediates proapoptotic signaling from inducers of apoptosis such as TGF-β1, whereas p38β relays survival signaling from prosurvival factors including VEGF, and that TGF-β1 induces endothelial cell apoptosis by converting VEGF signaling from the prosurvival p38β to the proapoptotic p38α.

Materials and Methods

Materials

Human purified or recombinant TGF-β1, recombinant human VEGF, and antibodies to p38α, p38γ, and p38δ were purchased from R&D Systems; antibodies to human cleaved caspase 3 and cleaved PARP from Cell Signaling Technologies; mouse and rabbit nonimmune immunoglobulin G (IgG) from Sigma-Aldrich; and antibodies to p38α, α-tubulin, and cyclin E from Santa Cruz Biotechnology, Inc., Human recombinant fibroblast growth factor-2 (FGF-2) was purchased from Gibco BRL; antibody to active p38 from Promega Corp.; monoclonal antibody to p38β from Zymed Laboratories; recombinant p38α from R&D Systems; p38 β from Biovision; and p38γ from Abnova.

Cells and media

Bovine capillary endothelial cells (BCE) were as described (28) and used at passages 6 to 15. Human umbilical vein endothelial (HUVE; Clonetics) cells were grown in medium (EBM2; Clonetics) containing 2% fetal calf serum (FCS) and the growth supplements provided by the company, and were used at passages 3 to 5. BCE or HUVE cells were starved overnight in their medium supplemented with 0.5% donor calf serum (DCS) or FCS, respectively, after which either TGF-β1 (1 ng/mL) or VEGF (30 ng/mL) or FGF-2 (10 ng/mL) was added and incubation was continued for the indicated time. The time of addition of TGF-β1 was considered as time 0. Western blotting was carried out as described (19). Bromodeoxyuridine (BrdU) uptake was carried out as described (19).

**Immunoprecipitation.** Cells were lysed in 10 mmol/L Tris-HCl pH 7.4 containing 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Pefabloc (Roche), 1 mmol/L leupeptin, 1 mmol/L Na3VO4, and 2 mmol/L CaCl2, (plus 100 μmol/L peryoxanate for phosphorylation studies). One hundred micrograms of cell extract protein was precleared at 4°C for 30 minutes with 0.5 μg of nonimmune IgG coupled to 10 μL of protein A+G agarose beads (Santa Cruz Biotechnology). Precleared extracts were centrifuged for 60 seconds at 300 × g, and the supernatant was immunoprecipitated overnight at 4°C with 10 μL of protein A+G agarose beads and 0.75 μg of antibody per 100 μg of protein. After washing 3 times with lysis buffer, the beads were boiled in reducing Laemmli buffer for 5 minutes and loaded onto SDS/PAGE gels.

**siRNA transfection.** Subconfluent HUVE cells were transiently transfected as described (19) and used for the experiments 48 hours after transfection.

**Reverse transcription PCR.** The following primers were synthesized by IDT DNA technologies based on the published sequences: glyceraldehyde-3-phosphate dehydrogenase: (forward 5′-CCC ACT CTT CCA CCT TCG-3′; reverse 5′-TCC TTG GAG GCC ATG TAG GCC AT-3′); p38α: (forward 5′-GCA GGG ACC TTC TCA TAG AT-3′; reverse 5′-GAG GGA TAG CCT CAG ACC-3′); p38β: (forward 5′-CTG CAA GGA AAG GCC CTC-3′; reverse 5′-CAG GCA ATG CCT CAC TGC-3′); p38γ: (forward 5′-GAT TAC TGG GAA GAT CTT G-3′; reverse 5′-CTG CAC AGA GCC GTC TCC-3′); p38δ: (forward 5′-GAC ACT CTT CAA GGG CAA G-3′; reverse 5′-GCC ATC AAT CAC TGC AGC-3′). cDNA was synthesized from 1 μg of total RNA with SuperScript II RT (Invitrogen) and oligo-dT 3′ primer. cDNA (2 μL) was amplified by PCR as described.

**Proximity ligation assay.** HUVE cells grown on gela-}

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and 40 μL per well of collagen was added. After incubation at 37°C for 30 minutes, 100 μL of medium supplemented with 0.5% FCS and 1 ng/mL of TGF-β1 was added, and the plates were incubated at 37°C (30). TGF-β1 (1 ng/mL) was added every other day. After 5 to 7 days, the culture medium was removed; the cultures were washed with PBS, stained with toluidine blue, and photographed under an inverted microscope. The number of tube-like structures forming anastomoses was counted as described (20).

**Statistical analysis**

The Student t tests on the equality of means were carried out using Stata 8.

**Results**

We have previously shown that TGF-β1 induces endothelial cell apoptosis in vitro and in vivo through VEGF/VEGFR-2 activation of p38MAPK (19, 20). In the absence of TGF-β1, VEGF activates p38MAPK but protects endothelial cells from apoptosis (19). Therefore, our finding raised the question: how does TGF-β1 convert VEGF signaling from anti- to proapoptotic? Because p38MAPK exists in 4 isoforms with different biological functions, we hypothesized that TGF-β1 shifts VEGF activation of p38MAPK from 1 isoform to another.

To investigate this hypothesis, we characterized endothelial cell expression of the p38 isoforms, and the effect of TGF-β1 and VEGF on their activation. By reverse transcriptase PCR (RT-PCR) endothelial cells expressed p38α, β, and γ mRNAs, whereas p38δ mRNA was barely detectable (Fig. 1A). Western blotting with p38MAPK isoform-specific antibodies showed expression of the corresponding proteins (Fig. 1B and C).

Neither VEGF nor TGF-β1 treatment of endothelial cells significantly altered p38α, β, or γ expression (Fig. 1D), suggesting that the activity of these kinases is regulated at posttranscriptional level. Isoform-specific antibodies to phosphorylated p38 do not exist because the isoforms’ phosphorylation sequences are identical. Therefore, we immunoprecipitated extracts of TGF-β1- or VEGF-treated cells with antibodies to the individual p38 isoforms and analyzed the immunoprecipitates by Western blotting with an antibody that recognizes all phosphorylated isoforms. The results (Fig. 2A) showed that untreated endothelial cells had no active p38δ but showed comparable levels of active p38β and p38γ. Treatment with TGF-β1 selectively activated p38α. Conversely, VEGF selectively activated p38β. FGF-2, which protects endothelial cells from apoptosis, strongly upregulated p38β activation; in contrast, UVB irradiation, which induces apoptosis, activated p38δ.

Although the anti-p38δ and p38β antibodies did not show cross-reactivity by Western blotting (Fig. 1C), they might cross-react by immunoprecipitation. Therefore, we carried out reverse experiments in which cell extracts were immunoprecipitated with antibody to phospho-p38 and the immunoprecipitates analyzed by Western blotting with antibodies to p38δ or p38β. The results (Fig. 2B) showed that treatment with FGF-2 or VEGF selectively activated p38β, whereas TGF-β1 or UVB irradiation activated p38δ. Western blotting analysis of caspase 3 activation, a marker of apoptosis, showed that TGF-β1 and UVB induced cell death, whereas FGF-2 and VEGF had no such effect (Fig. 2B). Therefore, these findings suggested that in endothelial cells p38δ mediates apoptotic signaling, whereas p38β relays prosurvival signaling. Our previous studies (19, 20) have shown that in endothelial cells TGF-β1 activation of p38MAPK is abolished by downregulation of VEGFR-2, showing that endothelial cell VEGF mediates p38MAPK activation by TGF-β1. In addition, TGF-β1 does not induce apoptosis in endothelial cells that do not express VEGF in response to TGF-β1 (19). Because in the absence of TGF-β1 VEGF activates p38β, our results suggested that TGF-β1 induces endothelial cell apoptosis by shifting VEGF activation of p38MAPK from the β to the α isoform.

The apoptotic effect of TGF-β1 is rapid (3–12 hours) and followed by refractoriness of the surviving cells to TGF-β1 induction of apoptosis up to 96 hours (20). We found that TGF-β1 treatment of endothelial cells for 72 to 96 hours resulted in increased BrdU uptake and cyclin E expression between 24 and 96 hours (Figs. 3 and 4), coincident with the formation of capillary-like structures (Fig. 3; refs. 20, 7). These structures are likely representative of precapillary...
TGF-β and 4). The level of BrdU uptake induced by TGF-β (Supplementary Fig. S1). A low level of apoptosis (caspase-3 rapid and transient induction of apoptosis, followed by hours, a condition that induces endothelial cell apoptosis cells grown in starvation medium (0.5% serum) for 72 to 96 comparable with that induced by 10% FCS and occurred in transition as the cells did not express isoform(s) other than the one that mediates apoptosis. and do not reflect endothelial to mesenchymal trans- cordons and do not re
ditions, we hypothesized that the
findings, we used the
proximity in situ ligation assay (29) with antibodies to p38MAPK followed by Western blotting with p38α or p38β antibodies. The results (Fig. 4A and B) showed that p38α activation occurred between the initial 6 to 24 hours of treatment, coincident with apoptosis, while p38β activation decreased. Subsequently, the level of active p38α decreased, and p38β activation increased between 48 and 96 hours, concurrently with cell proliferation (Fig. 3 and 4) and refractoriness to TGF-β1 induction of apoptosis (20). Activation of p38α—to a level lower than at 6 hours—also occurred at 72 to 96 hours, coincident with the low level of caspase-3 activation observed at this time of incubation.

To confirm these findings, we used the in situ proximity ligation assay (29) with antibodies to p38α or p38β together with phospho-p38 antibodies, which allowed the detection of phosphorylated p38α and p38β in individual cells. The results showed that cells treated with TGF-β1 for 6 hours stained positively for p38α but not for p38β. Conversely, TGF-β1 treatment for 72 hours resulted in activation of p38β but not of p38α (Fig. 5). Thus, altogether these results indicated that p38β mediates TGF-β1 induction of apoptosis, whereas p38β is associated with TGF-β1–induced cell proliferation and refractoriness to apoptosis.

We therefore used siRNAs to selectively downregulate the p38α, β, and γ isoforms and characterized their effect on apoptosis. Transient transfection with siRNA to one p38 isom connected, showing

Figure 2. Control of p38 isoform activation in endothelial cells. A, endothelial cells were incubated with either FGF-2 (10 ng/mL) or VEGF (30 ng/mL) or TGF-β1 (1 ng/mL) for 6 hours, or irradiated with UVB (20 mJ/cm²). Cell extracts were immunoprecipitated with antibodies to p38α, β, or γ and analyzed by Western blotting with the corresponding antibodies and with antibody to phospho-p38 (p[p]p38). B, endothelial cells were treated with the indicated reagents as described above. Cell extracts were immunoprecipitated with antibody to phospho-p38 and analyzed by Western blotting with antibodies to p38α or p38β (bottom). Extracts were also analyzed by Western blotting for cleaved caspase-3 (cl-Csp3), an apoptosis marker, and p38 activation (p[p]p38) (top). ERK1/2, loading control.

Consistent with our previous findings (19, 20), Western blotting analysis with an antibody that recognizes all phosphorylated p38 isoforms showed that TGF-β1 induced p38MAPK activation between 3 and 9 hours of treatment (Fig. 4A), concomitantly with the onset of apoptosis, and subsequently between 72 and 96 hours, a time when addition of TGF-β1 fails to induce apoptosis (20) and cell proliferation occurs (Figs. 3 and 4). This observation suggested that the early induction of p38MAPK activation (3–6 hours) involves the α isoform, which is associated with apoptosis, and the late activation (72–96 hours) entails the β isoform, which mediates survival signaling. Therefore, we analyzed the cell extracts by immunoprecipitation with antibody to phospho-p38MAPK followed by Western blotting with p38α or p38β antibodies. The results (Fig. 4A and B) showed that p38α activation occurred between the initial 6 to 24 hours of treatment, coincident with apoptosis, while p38β activation decreased. Subsequently, the level of active p38α decreased, and p38β activation increased between 48 and 96 hours, concurrently with cell proliferation (Fig. 3 and 4) and refractoriness to TGF-β1 induction of apoptosis (20). Activation of p38α—to a level lower than at 6 hours—also occurred at 72 to 96 hours, coincident with the low level of caspase-3 activation observed at this time of incubation.

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The morphologic analysis of the siRNA-transfected cells showed effects consistent with the biochemical characterization of apoptosis (Fig. 6C). As described (7, 20), confluent endothelial cells treated with TGF-β1 for 72 to 96 hours formed structures that resemble precapillary cords (Fig. 6C, a and b). However, TGF-β1–treated cells transfected with p38α siRNA retained a confluent monolayer with no apparent morphologic changes (Fig. 6C, c). In contrast, transfection with p38β or p38γ siRNAs caused massive cell death resulting in sparse, disrupted cord-like structures (Fig. 6C, d and e). Notably, downregulation of these p38 isoforms blocked the proliferative effect of TGF-β1 that follows the transient induction of apoptosis (Figs. 3 and 4), and induced massive apoptosis at a time of TGF-β1 treatment (72–96 hours) when endothelial cells are refractory to TGF-β1 induction of apoptosis (20).

To investigate the relative contribution of p38α and p38β to blood vessel formation, we used an in vitro angiogenesis assay (Fig. 7). For this purpose, HUVE cells were either mock transfected or transiently transfected with siRNAs to the α, β, or γ isoforms of p38 and grown in 3D collagen gels in the presence of 1 ng/mL of TGF-β1. Within 5 to 7 days, mock transfected cells formed a network of cord-like structures as described (30). In contrast, with cells transfected with the siRNAs to the p38MAPK isoforms this effect was dramatically blocked. Cells transfected with p38α siRNA showed very rudimentary cord-like structures larger than those obtained with mock-transfected cells and consisting of flattened cells that in several areas maintained a confluent monolayer. Conversely, cells transfected with siRNAs to p38β or p38γ showed sparse, disrupted capillary-like structures that failed to form a network (Fig. 7). Numerous areas of the collagen gels only contained isolated clusters of globular cells.

Thus, the p38MAPK isoforms have opposing roles in TGF-β1 induction of in vitro angiogenesis, which mirror their effects on endothelial cell apoptosis and survival. Because p38α activation by TGF-β1 is mediated by VEGF (19, 20), which in the absence of TGF-β1 activates p38β,
isoform of p38MAPK mediates prosurvival signaling induced 3 times with comparable results. Our results show that TGF-β1 induces endothelial cell apoptosis through VEGF/VEGFR-2–mediated activation of p38MAPK (19). Because in the absence of TGF-β1, VEGF activates p38MAPK but protects endothelial cells from apoptosis, our finding suggested that TGF-β1 modifies VEGF signaling from anti- to proapoptotic. The data reported in this article show that TGF-β1 induces endothelial cell apoptosis by shifting VEGF activation of p38MAPK from the β to the α isoform. In endothelial cells, the β isoform of p38MAPK mediates prosurvival signaling induced by growth factors such as VEGF and FGF-2, which protect endothelial cells from apoptosis. Conversely, p38α mediates apoptotic signaling from inducers of endothelial cell apoptosis such as TGF-β1 and UVB. p38γ also mediates endothelial cell survival, but is not controlled by VEGF or TGF-β1.

Numerous studies implicate p38MAPK in apoptosis. In endothelial cells, p38MAPK activation mediates apoptosis induction by a variety of agents (24–27, 32–34), and VEGF blocks endothelial cell apoptosis by decreasing p38MAPK phosphorylation (27, 35). However, the p38MAPK isoforms involved in endothelial cell apoptosis have not been characterized. In cell types other than endothelial cells, p38MAPK activation in response to a variety of stimuli induces either proliferation or growth arrest/apoptosis, depending on the cell type (21, 36–38). The specific p38 isoforms involved in these processes remain largely unidentified. In human fibroblasts activation of p38γ, but not of other p38 isoforms, is required for induction of G2 arrest by γ-irradiation (39), suggesting that the individual p38 isoforms can have different effects on cell fate. Both p38α and β have been implicated in the pathogenesis of myocardial hypertrophy, where they may play different roles; p38β being more potent in inducing hypertrophy, and p38α in other functions such as apoptosis (40). Similarly, p38α but not p38β is required for ischemic preconditioning of the myocardium (41). Our study is the first to identify opposing roles for the α and β isoforms of p38MAPK in the control of endothelial cell survival and apoptosis.

Our data also show several features of the mechanism by which TGF-β1 controls endothelial cell death and proliferation. Under most culture conditions, TGF-β1 is a potent inhibitor of endothelial cell proliferation and migration, and an inducer of apoptosis (42, 43). However, TGF-β1 also stimulates endothelial cell proliferation in vitro and in vivo (10, 43, 44). We found that the apoptotic effect of TGF-β1 our results show that TGF-β1 converts VEGF signaling from activation of the prosurvival p38β to the proapoptotic p38α.

Discussion

We have previously shown that TGF-β1 induces endothelial cell apoptosis through VEGF/VEGFR-2–mediated activation of p38MAPK (19). Because in the absence of TGF-β1, VEGF activates p38MAPK but protects endothelial cells from apoptosis, our finding suggested that TGF-β1 modifies VEGF signaling from anti- to proapoptotic. The data reported in this article show that TGF-β1 induces endothelial cell apoptosis by shifting VEGF activation of p38MAPK from the β to the α isoform. In endothelial cells, the β isoform of p38MAPK mediates prosurvival signaling induced by growth factors such as VEGF and FGF-2, which protect endothelial cells from apoptosis. Conversely, p38α mediates apoptotic signaling from inducers of endothelial cell apoptosis such as TGF-β1 and UVB. p38γ also mediates endothelial cell survival, but is not controlled by VEGF or TGF-β1.

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![Figure 6.](image-url) Control of endothelial cell apoptosis and survival by p38α, β, and γ. A, Western blotting analysis of p38 isoform expression in HUVE cells transfected with siRNAs to either p38α or β or γ. A blot with an antibody to all p38 isoforms (Tot. p38) is shown as a loading control. B, HUVE cells transfected with siRNAs to either p38α or β or γ, or with a mixture thereof (Mix), or mock transfected (control) and incubated with TGF-β1 (1 ng/mL) or control medium for 6 hours were analyzed by Western blotting with antibody to cleaved PARP (PARP p85) or to total PARP as a loading control. C, confluent HUVE cells grown in gelatin-coated dishes and either non transfected and untreated (a) or transfected with transfection reagent alone (b) or siRNAs to either p38α (c) or β (d) or γ (e). The cells were photographed after 96 hours incubation in the absence (a) or presence (b–d) of TGF-β1 (1 ng/mL). Magnification, ×100. Histogram. The results were quantitated as described (20). Mean ± SE of triplicate samples are shown. *, P < 0.05 (sample vs. a). These experiments were repeated 3 times with comparable results.

![Figure 7.](image-url) In vitro angiogenesis assay in 3D collagen gel. A, HUVE cells transfected with the indicated siRNAs or mock transfected (-siRNA), and grown in 3D collagen gels for 6 days in the presence of TGF-β1 (1 ng/mL). Magnification, ×100. B, results of the experiments shown in A quantitated as described under Materials and Methods. Mean ± SE of triplicate samples are shown. *, P < 0.05 (sample vs. a). These experiments were repeated 3 times with comparable results.
Control of Endothelial Cell Apoptosis by p38MAPK Isoforms

on endothelial cells is rapid and transient, and is followed by a long period during which the surviving cells are refractory to apoptosis induction by TGF-β1 (20) and proliferate. These opposing effects of TGF-β1 are mediated by the selective activation of 2 different p38 isoforms. The rapid induction of apoptosis results from downregulation of p38β and upregulation of p38α activation. Subsequently, down-regulation of p38α and parallel, sustained increase in p38β activation provide survival signaling. On the basis of our data, we cannot conclude whether TGF-β1 induction of endothelial cell proliferation is also mediated by p38β because siRNA-mediated downregulation of p38β expression results in rapid and massive apoptosis. Our previous findings that TGF-β1 induces prolonged upregulation of endothelial cell VEGF and FGF-2 expression and activates extracellular signal-regulated kinase (ERK)1/2 by a VEGF/VEGFR-2-dependent mechanism (19) suggest that the proliferative effect of TGF-β1 could be mediated by ERK1/2, while the sustained activation of p38β provides endothelial cells with survival signaling. Thus, TGF-β1 controls both endothelial cell apoptosis and survival through the selective activation of the α and β isoforms of p38MAPK, respectively.

The effect of TGF-β1 on endothelial cells is mediated by 2 type-I receptors (TGF-βR1): activin receptor–like kinase (ALK)-5, the predominant type I receptor that mediates TGF-β1 responses, and ALK-1, whose expression is restricted to endothelial cells. ALK-5 and ALK-1 mediate TGF-β1 signaling through distinct Smad proteins: Smad2/Smad3 and Smad1/Smad5, respectively (11). Although TGF-β1 primarily activates Smad2 and Smad3, in primary endothelial cells it also activates Smad1 and Smad5 through a heterodimeric receptor complex consisting of ALK5 and ALK1 (45). The ALK5 and ALK1 pathways mediate opposing effects; ALK5 inhibits, whereas ALK1 stimulates cell migration and proliferation (46). In addition, ALK5, but not ALK1, mediates TGF-β1 induction of endothelial cell apoptosis (47). However, ALK-1/Smad1 signaling mediates TGF-β1 induction of VEGF expression in endothelial cells (18), which is required for TGF-β1 induction of apoptosis (19). Based on our data, we cannot conclude whether ALK1 and ALK5 activate different p38 isoforms. It is tempting to speculate that signaling by both receptors is required for TGF-β1 induction of apoptosis, and that downregulation of ALK5 is responsible for the ensuing cell proliferation (Fig. 4; ref. 20). The relative contribution of TGFβRIs to activation of the different isoforms of p38MAPK and control of endothelial cell apoptosis warrants further investigation.

Our conclusions are partly based on results of siRNA-mediated gene silencing experiments. It has been reported that siRNAs can inhibit angiogenesis by a nonspecific effect mediated by toll-like receptor 3 (TLR3), a double-stranded RNA receptor that generates apoptotic signaling in endothelial cells (48, 49). This effect requires siRNAs more than 21 nucleotides in length, and has been obtained with nonchemically modified molecules. We used 19-bp siRNAs with dinucleotide 3′ DNA overhangs, a chemical modification to prevent nonspecific effects. Indeed, in our studies, we did not observe nonspecific apoptotic effects or p38MAPK activation induced by our siRNAs. Our VEGF-1 and VEGFR-2 siRNAs did not induce apoptosis (19, 20), nor did our siRNAs to p38α or a mixture of siRNAs to p38β, and γ (Fig. 6B). Similarly, our siRNAs to VEGF-1 and VEGFR-1, or control siRNA to lamin A did not induce p38MAPK activation (19). Therefore, our siRNAs did not have nonspecific effects on p38MAPK activation or endothelial cell apoptosis mediated by TLR3.

Our results are in contrast with previous data showing that pharmacologic inhibition of p38MAPK blocks endothelial cell apoptosis (19, 27, 35). Because p38β and p38γ mediate survival signaling, their inhibition should result in apoptosis. This discrepancy is explained by the fact that all currently available inhibitors of p38MAPK inhibit both p38α and p38β, and therefore abolish both pro- and antipapoptotic signaling. In addition, the apoptotic effect of p38α activation seems to be prevalent. Indeed, siRNA-mediated down-regulation of all the p38 isoforms expressed by endothelial cells blocks TGF-β1 induction of apoptosis as effectively as downregulation of p38α alone. In contrast, selective inhibition of p38β or p38γ expression results in apoptosis (Fig. 6B and C).

On the basis of our data, we cannot conclude whether the proliferative effect of TGF-β1 on endothelial cells is mediated by VEGF or other endothelial cell-derived growth factors. TGF-β1-induced upregulation of VEGF lasts at least 24 h (19), a timing coincident with the onset of cell proliferation. Although TGF-β1 downregulates endothelial cell expression of VEGFR-2, this effect does not exceed 40–50% (13, 14). In addition, TGF-β1 upregulates endothelial cell expression of FGF-2 (19), which can also provide a mitogenic stimulus. Therefore, both VEGF and FGF-2 could mediate TGF-β1 induction of endothelial cell proliferation through sustained activation of the ERK1/2 or other signaling pathways.

The coordinated mechanism of control of endothelial cell apoptosis, survival, and proliferation by TGF-β1—VEGF activation of p38α and β has profound effects on in vitro angiogenesis. Downregulation of p38α- and p38β-mediated apoptosis blocks the formation of capillary-like structures in 3D collagen gel. Similarly, induction of excess apoptosis by downregulation of p38β—mediated survival signaling blocks endothelial cell sprouting and organization into a capillary-like network. These findings are consistent with our previous observation that inhibition of endothelial cell apoptosis blocks angiogenesis in vitro and in vivo (20).

We have previously shown that VEGF mediates TGF-β1 induction of endothelial cell apoptosis through activation of p38MAPK (19). This conclusion was based on our findings that inhibition of VEGF or downregulation of VEGFR-2 blocks TGF-β1 activation of p38MAPK, and inhibition of p38MAPK expression abrogates TGF-β1 induction of apoptosis (19). Our data show that VEGF activates the pro-survival p38β, whereas TGF-β1 activates the proapoptotic p38α. Because TGF-β1 activates p38β through VEGF, which in the absence of TGF-β1 activates p38β, our findings.
show that TGF-β1 shifts VEGF signaling from p38β to p38α.

Our results therefore generate the following model for the control of endothelial cell apoptosis and survival by TGF-β1 and VEGF (Fig. 8). In the absence of TGF-β1, VEGF binding to VEGFR-2 induces endothelial cell activation of p38β and cell survival (Fig. 8, left). TGF-β1 induces endothelial cell expression of VEGF and switches VEGF/VEGFR-2–activated signaling to the proapoptotic p38α. This effect involves crosstalk between TGF-β1 and VEGF signaling pathways that can occur at multiple levels upstream of p38α MAPK. VEGF/VEGFR-2–mediated activation of p38α is necessary for TGF-β1 induction of endothelial cell apoptosis; however, parallel TGF-β1–initiated signaling acting in concert with p38α may also be required. Subsequently (Fig. 8, right), the cells become refractory to TGF-β1, p38α activation is downregulated and p38β activation upregulated. This effect results in abrogation of apoptosis and increased prosurvival signaling.

The signaling mechanism(s) that control the selective activation of the α or β isoform of p38 are largely unknown, as is the crosstalk between TGF-β1 and VEGF signaling that mediates the shift of VEGF activation of p38 from the β to the α isoform. Understanding these mechanisms can shed light on the complex control of vascular endothelial cell survival and death during angiogenesis. Importantly, our findings provide indications for the development of novel pharmacologic approaches to inhibit tumor angiogenesis. We found that, whereas the effect of TGF-β1 on apoptosis is transient (Fig. 4) and followed by refractoriness of the cells to TGF-β1 induction of apoptosis (20), inhibition of p38β and/or p38γ signaling results in sustained induction of apoptosis by TGF-β1 (Fig. 6). Presently, all synthetic inhibitors of p38 block both the α and the β isoforms. Novel inhibitors that selectively inhibit p38β activity (or activation) could block the prosurvival effect of TGF-β1 (and VEGF) on endothelial cells which follow apoptosis induction. These inhibitors could thus transform the transient apoptotic effect of TGF-β1 into a sustained, massive effect. Mice genetically deficient in p38β show no major phenotype if unchallenged (50), indicating that selective inhibition of p38β would represent a feasible and safe approach to antitumor angiogenesis therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


Control of Endothelial Cell Apoptosis by p38MAPK isoforms


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