NF-κB activation in endothelial cells is critical for the activity of angiostatic agents

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Abstract

In tumor cells, the transcription factor NF-κB has been described to be antiapoptotic and proproliferative and involved in the production of angiogenic factors such as vascular endothelial growth factor. From these data, a protumorigenic role of NF-κB has emerged. Here, we examined in endothelial cells whether NF-κB signaling pathway is involved in mediating the angiostatic properties of angiogenesis inhibitors. The current report describes that biochemically unrelated agents with direct angiostatic effect induced NF-κB activation in endothelial cells. Our data showed that endostatin, anginex, angiostatin, and the 16-kDa N-terminal fragment of human prolactin induced NF-κB activation in endothelial cells in both cultured human endothelial cells and in vivo in a mouse tumor model. It was also found that NF-κB activity was required for the angiostatic activity, because inhibition of NF-κB in endothelial cells impaired the ability of angiostatic agents to block sprouting of endothelial cells and to overcome endothelial cell anergy. Therefore, activation of NF-κB in endothelial cells can result in an unexpected antitumor outcome. Based on these data, the current approach of systemic treatment with NF-κB inhibitors may therefore be revisited because NF-κB activation specifically targeted to endothelial cells might represent an efficient strategy for the treatment of cancer. [Mol Cancer Ther 2009;8(9):2645–54]

Introduction

Inhibition of angiogenesis is a promising therapeutic strategy in the fight against cancer. Many angiostatic approaches are based on neutralizing angiogenic proteins, such as vascular endothelial growth factor, basic fibroblast growth factor (bFGF), and platelet growth factor, or blocking of their receptors by tyrosine kinase inhibitors (1, 2). Other approaches work through direct targeting of endothelial cells, resulting in inhibition of migration, proliferation, survival, or maturation of endothelial cells (3). Although the best developed angiostatic strategies are based on vascular endothelial growth factor targeting, considerable progress has been made in the discovery and development of agents that directly interfere with tumor endothelial cells. However, a clear and precise view on the mechanisms of action of these angiostatic agents is still lacking and necessary for the design of improved angiostatic compounds.

The NF-κB/Rel family of transcription factor includes five proteins, p50, p52, p65 (or RelA), RelB, and c-Rel, which exist as homodimers or heterodimers. The most common NF-κB heterodimer is composed of p50 and p65. In resting cells, NF-κB is sequestered in the cytoplasm by its association with proteins belonging to the IκB inhibitor family. Stimuli such as the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin-1 or bacterial products such as lipopolysaccharide can activate NF-κB. In the canonical pathway, these stimuli trigger activation of an IκB kinase complex, which in turn phosphorylates NF-κB inhibitors (IκB; ref. 4). This phosphorylation step leads to the ubiquitination and subsequent degradation of IκB by the proteasome. The NF-κB complex translocates into the nucleus where it binds to κB enhancers present in the regulatory regions of target genes and where it activates transcription (5). Many proteins encoded by NF-κB target genes participate in the immune response. However, NF-κB is also involved in the activation of genes involved in proliferation, apoptosis, and angiogenesis. For this reason, NF-κB is considered to be connected with multiple aspects of oncogenesis (6).

In tumor cells, NF-κB is generally seen as an antipoptotic factor because it induces antiapoptotic genes that block the caspase cascade or antiapoptotic members of the Bcl family, described to inhibit cytochrome c release. Therefore, activation...
of NF-κB in cancer cells by chemotherapy or radiation therapy is often associated with the acquisition of resistance to apoptosis. This has emerged as a significant impediment to effective cancer treatment. In combination with chemotherapy, inhibitors of the NF-κB pathway were recently successfully used as treatment against cancer (7). However, in contrast to the negative effects of NF-κB activation, recent reports suggest that, in certain situations, NF-κB can promote apoptosis and may be viewed as a tumor suppressor gene (8). NF-κB has been described to induce expression of several proapoptotic molecules such as Bcl-Xs, Bax, Fas, or Fas ligand (9) and blockade of NF-κB predisposes murine skin to squamous cell carcinoma (10). It has been shown that the dual activity of NF-κB, as an inhibitor or activator of apoptosis, may depend on the relative levels of p65 and c-Rel subunits; the later has been described to induce apoptotic signal in response to TNF-related apoptosis-inducing ligand (11). These data clearly illustrate the dual activity of NF-κB and the complexity of the systemic application of broad-spectrum NF-κB inhibitors for the treatment of cancer.

The current report describes the role of NF-κB in angiostatic signaling in endothelial cells. We show that various angiostatic agents induce NF-κB activation in endothelial cells in both cultured endothelial cells in vitro and tumors in vivo. In addition, we show that NF-κB inhibition impairs the ability of angiostatic agents to block sprouting of endothelial cells and to overcome endothelial cell anergy. Therefore, our data illustrate that activation of NF-κB is a common pathway activated by angiostatic agents in endothelial cells and show that NF-κB activation in endothelial cells contributes to a nondirect antitumor activity via angiogenesis inhibition and to overcoming endothelial cell anergy. Moreover, they suggest that selective NF-κB activation in endothelial cells might be an efficient strategy for the treatment of cancer, thereby raising a cautionary note against the broad and systemic use of pharmaceutical agents that block NF-κB for cancer therapy.

Materials and Methods

Cell Cultures and Reagents

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously (20). Cells between passages 1 and 3 were used for experiments. Anginex and recombinant 16K hPRL were produced and purified as described previously (12, 13). Anginex is a 33-mer designer peptide with antiangiogenesis and antitumor activity. The structure of it is based on the three-dimensional structure of the chemokines platelet factor-4 and interleukin-8 (14).

NF-κB Binding Activity

HUVECs were stimulated with the different compounds as indicated. The p65 DNA-binding activity of NF-B was quantified by ELISA by means of the EZ Transcription Factor kit NF-κB p65 (Pierce, Thermo-Scientific) according to the manufacturer’s instructions on nuclear extract. Nuclear protein cell extracts were obtained as described previously (15). Protein concentrations were determined by the Bradford method using the Bio-Rad protein assay reagent. NF-B binding to the target oligonucleotide was detected by incubation with primary antibody specific for the activated form of p65, visualized by anti-IgG horseradish peroxidase conjugate and developing solution, and quantified by chemiluminescence with a luminometer. Specificity was established by incubation with a wild-type consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGG-3′), which competes with the substrate for binding. All samples were analyzed in duplicate.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays were done as described previously (16). Nuclear extract samples containing 5 μg protein were mixed with the 32P-labeled probe (sequence available upon request). The complexes were separated on a polyacrylamide gel. To confirm specificity, competition assays were done with a 50-fold excess of unlabeled wild-type and nonspecific probes. For supershifting experiments, 1 μg of each antibody (p50 or p65; both from Santa Cruz Biotechnology) was incubated with the extracts for 30 min on ice before the addition of the 32P-labeled probe.

Mouse Tumor Model and lacZ Staining

C57BL/6 α-B-dependent lacZ transgenic mice, line 189-4 (17), 10 to 15 weeks old, were s.c. inoculated on the right flank with 10⁵ B16F10 melanoma cells. These transgenic mice contain the p105 promoter upstream of the lacZ reporter gene, which causes production of β-galactosidase in response to the activation of NF-κB. At day 11, these mice were treated with anginex (10 mg/kg), endostatin (10 mg/kg), or TNF-α (5 μg/kg). Twenty-four hours after treatment, mice were sacrificed and tumors were resected and snap frozen immediately.

Tissue sections were processed for β-galactosidase activity detection as described previously (18). In short, tissues were fixed in ethanol, dehydrated in xylene, and then embedded by three passages at 44°C in low melting point paraffin (Merck, VWR). For β-galactosidase activity detection, paraffin sections (7 μm) were incubated in X-Gal solution at 32°C for 16 h as described previously.

Dual staining for PECAM-1 and β-galactosidase has been done with a rat anti-mouse PECAM-1 (Becton Dickinson) and an anti-rat secondary antibody coupled to the peroxidase (blue staining) and a rabbit anti-β-galactosidase (kindly given by J.F. Nicolas, Institut Pasteur) identified by an anti-rabbit secondary antibody coupled to the alkaline phosphatase (red staining).

RNA Extraction and Quantitative Reverse Transcription-PCR

Total RNA was extracted from tumor using the RNaseasy kit (Qiagen) according to the manufacturer’s instructions. Synthesis of cDNA was done starting with 1 μg total RNA, which was reverse transcribed with the Transcriptor First-Strand cDNA Synthesis kit (Roche Clinical Laboratories) according to the manufacturer’s instructions. The resulting cDNA (300 pg) was used for real-time PCR with the iQSYBR Green PCR Supermix (Bio-Rad). Thermal cycling was done on an ABI PRISM 7700 Sequence Detection System apparatus.
(Applied Biosystems). For all reactions, negative controls were run with no template present, and random RNA preparations were also subjected to sham quantitative reverse transcription-PCR (no reverse transcription) to verify lack of genomic DNA amplification. Relative transcript levels for each gene were obtained using the relative standard curve method and normalized with respect to the housekeeping gene cyclophilin B. Primers, the sequences of which are available upon request, were designed using the Primer Express software and selected to span exon-exon junctions to avoid detection of genomic DNA.

**Proliferation/Viability Assay**

HUVECs were seeded at 5,000 per well in fibronectin-coated flat-bottomed 96-well tissue culture plates in culture medium supplemented with 10% FCS. After pretreatment of 1 h with BAY1170-82 (10 μmol/L), cells were treated with 6.5 μmol/L anginex. After 3 days, viability was assessed by adding 10 μL WST-1 reagent (Roche) and by measuring absorbance at 450 nm according to the manufacturer's recommendations.

**Migration Assay**

Migration assays were carried out as described previously (19). Briefly, HUVEC cultures were grown to confluence in gelatin-coated 24-well tissue culture plates. A cross-shaped wound was made by scratching the monolayer with a plastic tip. Wounded monolayers were washed with PBS and incubated with complete culture medium supplemented with bFGF (10 ng/mL). The wound width was measured microscopically at four different places 0, 2, 4, 6, and 8 h after wounding the culture.

**Sprouting Assay**

Sprouting assays were carried out as described previously (20). HUVECs were suspended in culture medium and seeded on nonadherent plates. The suspended cells integrate into a single spheroid of defined size and cell number. After 24 h of incubation at 37°C, the spheroids were embedded in a collagen gel and treated with the different factors. Quantification was done by digital imaging and calculating length of the spheroid sprouts after 8 h of incubation. At least 10 spheroids per group were analyzed.

**Leukocyte Adhesion Assay**

Human peripheral blood leukocytes were isolated by Ficoll gradient centrifugation (Amersham) and labeled with 10 μmol/L 5-(6)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes) for 30 min at 37°C. Cells were washed twice with PBS and subsequently allowed to adhere to confluent endothelial cells for 1 h at room temperature. After removal of the nonadhered cells, the wells were trypsinized and harvested cells were fixed for 30 min with 1% paraformaldehyde and ICAM-1 was detected using a mouse anti-human ICAM-1 monoclonal antibody (MEM111; Monosan).

**Statistics**

All values are expressed as means ± SD. All experiments were done in triplicate and repeated at least three times. Comparisons between different treatments were assessed using the Student's t test.

**Results**

**Angiostatic Agents Induce NF-κB Activation in Endothelial Cells**

We have shown previously that the 16-kDa N-terminal fragment of human prolactin (16K hPRL) is a potent angiostatic and antitumor factor, acting by an apoptosis-inducing mechanism that requires activation of NF-κB in endothelial cells (16, 23, 24). To investigate whether this activity is a common feature of angiostatic agents, we tested several other angiostatic compounds with a direct effect on endothelial cells, anginex (12, 14), endostatin (25), and angiostatin (26), along with 16K hPRL. These agents specifically target endothelial cells but are otherwise biochemically unrelated. Cultured human endothelial cells (HUVEC) were treated for 1 and 4 h with these inhibitors. The p65 DNA-binding activity of NF-κB was quantified by ELISA by immobilization of a double-stranded DNA consensus sequence for p65. TNF-α, used as a positive control, induced a significant 2.7-fold up-regulation of NF-κB activity in HUVEC after 1 h as shown by a higher nuclear p65 DNA-binding activity (Fig. 1A). Interestingly, all four angiostatic molecules significantly activated NF-κB in endothelial cells after 1 and 4 h of stimulation (Fig. 1B-E). The level of activation for all four angiostatic agents was found to be comparable with that of TNF-α (2- to 3-fold induction). In contrast, bevacizumab/Avastin (1), a humanized monoclonal vascular endothelial growth factor neutralizing antibody, failed to activate NF-κB (Fig. 1F). All four angiostatic agents activated NF-κB at concentrations described previously to block angiogenesis in vitro (data not shown). To confirm these results, RF-24 immortalized human endothelial cells were treated with TNF-α and the four angiostatic molecules for 1 h. ELISA revealed that TNF induced a significant 4.0-fold up-regulation of NF-κB activity (Fig. 1G). As observed previously in HUVEC, all four angiostatic agents significantly activated NF-κB in RF-24.

We then focused on the dissection of the molecular action of anginex as a model angiostatic compound. We first quantified the activation of NF-κB in HUVEC by a concentration range of anginex. Anginex induced NF-κB activity in a concentration-dependent manner starting at 1.25 μmol/L as shown by ELISA (Fig. 2A). A time-course experiment was then done with HUVEC exposed to 6.5 μmol/L anginex, a concentration known to block proliferation, migration, and sprouting of endothelial cells. Anginex already activated p65 DNA binding after 30 min of stimulation (Fig. 2B). Peak levels of activation were observed at 60 min, after which NF-κB activation decreased to basal levels over a period of ~2 h. A significant activation was then observed after fixed with 1% paraformaldehyde and ICAM-1 was detected using a mouse anti-human ICAM-1 monoclonal antibody (MEM111; Monosan).
4 h of stimulation, suggesting a biphasic NF-κB activation. Interestingly, significant activation of NF-κB was still observed 24 h after stimulation with anginex. A similar kinetic of activation was observed for endostatin, angiostatin, and 16K hPRL (data not shown). These results suggest that NF-κB activation is a proximal event in the pathway activated by angiostatic agent in endothelial cells.

To further identify the NF-κB dimer activated by anginex, electrophoretic mobility shift assays were done. Nuclear extracts from HUVEC, stimulated with 6.5 μmol/L anginex for 60 min, were incubated with a 32P-labeled κB DNA sequence and subsequently run on acrylamide gel. Only one specific band corresponding to the κB/NF-κB complex was found to increase on treatment with anginex (Fig. 2C, lane 2) compared with the band obtained from untreated cells (lane 1). The presence of only band suggested that only one NF-κB dimer was activated by anginex. The specificity of the NF-κB complex detected was shown by its disappearance in the presence of 100-fold molar excess of unlabeled κB probe (lane 5) and its steadiness in the presence of an unlabeled mutated probe (lane 6). Furthermore, use of p50- and p65-specific antibodies (lanes 3 and 4) completely shifted the
NF-κB complex induced by anginex. Further experimentation may be required to analyze if other NF-κB subunits may also be activated by anginex. From these experiments, we concluded that anginex mainly activates the p50/p65 heterodimer in cultured endothelial cells.

**Angiostatic Agents Activate NF-κB in Tumor Endothelial Cells**

We next investigated whether NF-κB is activated in vivo in tumor endothelial cells on treatment with angiostatic agents. To that end, we used transgenic mice in which a NF-κB-responsive promoter element drives a lacZ reporter gene containing a nuclear localization sequence at its 5’ terminus (17). B16F10 melanoma cells were injected s.c., which gave rise after 11 days to a tumor of 500 to 1,000 mm³. Mice minus (17). B16F10 melanoma cells were injected s.c., which gave rise after 11 days to a tumor of 500 to 1,000 mm³. Mice were then treated with the angiostatic agents anginex or endostatin (10 mg/kg) or injected with TNF-α (5 μg/kg) as a positive control of NF-κB activation. Anginex and endostatin have been found previously to reduce B16F10 melanoma tumor growth and microvessel density with a maximum of effect between 5 and 20 mg/kg/d (22, 27). Twenty-four hours later, tumors were resected and the level of NF-κB was assessed by X-Gal staining of tissue sections. Interestingly, β-galactosidase activity was observed exclusively in tumors of treated mice with anginex or endostatin as well as in tumors of mice that were injected with the positive control TNF-α (Fig. 3A). β-Galactosidase immunoreactivity colocalized with the endothelial cell marker PECAM-1. Endothelial cells with β-galactosidase activity were distributed throughout the tumor with most activity observed in the outer rim of the tumor. Even if we cannot rule out the possibility that TNF-α activated NF-κB in tumor cells, no tumor cells were positive for β-galactosidase after TNF-α treatment because tumor cells did not harbor the β-lacZ reporter construct.

To sensitively quantify the level of NF-κB activation observed in tumor endothelial cells, quantitative real-time reverse transcription-PCR experiments using primers complementary to the lacZ mRNA sequence were done on mRNA extracted from treated and untreated tumors. A significant and strong increase of the lacZ mRNA was observed in tumors treated with anginex (mean 4.8-fold) and TNF-α (mean 4.4-fold), whereas endostatin led to a significant but lower enhancement of this mRNA (mean 2.0-fold; Fig. 3B). We then confirmed the NF-κB activation by analyzing the expression of a natural target of NF-κB: IκB-α (Fig. 3C). IκB-α is a well-described target gene of NF-κB, which is involved in a negative feedback loop. Consistently with the lacZ results, we found a significant increase of IκBα mRNA in tumor treated with anginex (mean 1.9-fold) and TNF-α (mean 1.3-fold). For endostatin, the increase was very moderate and not significant. This moderate increase of IκBα expression compared with the one observed for lacZ could reflect that basal expression of IκBα is ubiquitous (in tumor and nontumor cells) compared with the lacZ expression, which is restricted to the nontumor cells. Therefore, the increase of IκBα expression may have been masked by the basal level of IκBα expression. Together, these results show that NF-κB-dependent transcription is significantly induced in tumor endothelial cells after treatment with angiostatic agents.
Implication of NF-κB Activation in the Angiostatic Signaling

To assess the importance of NF-κB in the angiostatic signaling, we blocked NF-κB activation with BAY1170-82, a chemical inhibitor known to interfere with IκB kinase (28, 29). In HUVEC, 10 μmol/L BAY1170-82 efficiently inhibited activation of NF-κB treated with anginex (Fig. 4A). As reported previously, and as shown in Fig. 4B, anginex strongly reduced the proliferation of HUVEC (∼80%). Pretreatment for 1 h with the NF-κB inhibitor did not affect this decrease, suggesting a NF-κB–independent effect of anginex on endothelial cell proliferation. Endothelial cell migration was then investigated using the wound assay. As shown in Fig. 4C, anginex inhibited the wound closure by endothelial cells. This effect was already significant after 4 h of stimulation. Pretreatment of endothelial cells with the NF-κB inhibitor did not modify the anginex antimit migratory feature. Finally, the role of NF-κB in the sprouting of endothelial cells was investigated. In the absence of growth factors (e.g., bFGF), HUVEC spheroids embedded in a collagen gel showed very little sprouts. Stimulation of spheroids with bFGF significantly increased the sprout lengths (Fig. 5A). Interestingly, NF-κB inhibitor BAY1170-82 tended to increase the sprout length on nonstimulated spheroids. Anginex treatment completely reduced the sprouting of endothelial spheroids induced by bFGF (Fig. 5B). After treatment with anginex, the sprout length was similar to that observed in the absence of bFGF. Pretreatment of endothelial cells with the NF-κB inhibitor for 1 h before anginex treatment strongly abrogated the anginex-induced inhibition of sprouting. These results showed the role of NF-κB in the control of endothelial cell sprouting and suggested the NF-κB dependency of anginex inhibition of endothelial cell sprouting.

NF-κB Is Required for Angiostatic-Induced Reversal of Endothelial Cell Anergy

Angiogenesis inhibitors are also shown to display an indirect antitumor activity through reversal of endothelial unresponsiveness to inflammatory signals, a process called endothelial cell anergy. It has been shown that this activity, restoration of adhesion molecules such as ICAM-1 to normal levels, is present in many, if not all, angiostatic agents (22, 30). To determine whether NF-κB activation is involved in this process, we monitored peripheral blood leukocyte adherence on a surface of cultured endothelial cells. HUVECs treated for 24 h with anginex showed an increased capacity to bind leukocytes. Pretreatment with the NF-κB inhibitor BAY1170-82
blocked ~80% of this activity (Fig. 6A). Fluorescence-activated cell sorting analysis showed that whereas anginex up-regulated ICAM-1 expression on the endothelial cell surface, NF-κB inhibitor abolished this up-regulation significantly (Fig. 6B). Altogether, these results show that the NF-κB pathway in endothelial cells is involved in overcoming endothelial cell anergy induced by ongoing angiogenesis.

Discussion

Angiostatic agents are increasingly important in the clinical management of cancer. Many angiostatic agents interfere with the angiogenic process by blocking the proliferation/survival, the migration, or the maturation of endothelial cells. The molecular targets of these agents have only been partially discovered (3, 31, 32). A thorough understanding of the mechanism of action of angiostatic agents, mainly at the level of proximal signaling events, is still required for the development of efficient antitumor therapies. The current report describes that angiostatic agents with a direct activity on endothelial cells, such as 16K hPRL, anginex, endostatin, and angiostatin, activate NF-κB in endothelial cells.

NF-κB activation was observed in endothelial cells already after 30 min of stimulation with angiostatic agents. This time-course activation is comparable with what has been described previously for TNF-α in endothelial cells (33). These results suggest that the NF-κB pathway is an early event in response to binding of angiostatic agents to their receptors. The transgenic mice harboring a NF-κB-responsive promoter element driving a lacZ reporter gene provided the opportunity to evaluate NF-κB activation in vivo after treatment with angiostatic agents in tumor endothelial cells both spatially and quantitatively.

Twenty-four hours after treatment with anginex or endostatin, NF-κB was activated in tumor endothelial cells as measured by β-galactosidase activity and expression of lacZ and IkBα mRNA. To our knowledge, this is the first report illustrating activation of NF-κB in tumor endothelial cells after treatment with angiostatic agents. In this context, it is interesting to note that NF-κB is known to be activated by proinflammatory cytokines such as TNF-α, interleukin-1, and IFN-γ. These cytokines have antiangiogenesis activity as well. This is most well known for TNF-α (34) and IFN-γ.

Figure 4. Implication of NF-κB activation in the angiostatic signaling. A, NF-κB activity in HUVEC pretreated or not with BAY1170-82 (10 μmol/L) and stimulated for 48 h with anginex (6.5 μmol/L). Nuclear extracts were evaluated for the presence of NF-κB p65 by ELISA. Results are relative mean values of at least three experiments. *, P < 0.05, compared with untreated plus vehicle; #, P < 0.05, compared with anginex plus vehicle. B, HUVECs were pretreated or not with BAY1170-82 (10 μmol/L), stimulated for 48 h with anginex (6.5 μmol/L), and assessed for their ability to incorporate bromodeoxyuridine. Bromodeoxyuridine incorporation was measured by ELISA and presented as relative mean values of at least three experiments. *, P < 0.01, compared with untreated plus vehicle. C, migration of endothelial cells was determined by wound assays. Confluent HUVEC cultures were pretreated or not with BAY1070-82 (10 μmol/L), wounded, and then treated with anginex (6.5 μmol/L). Wound width is plotted as relative mean values of at least three experiments. *, P < 0.05; **, P < 0.01, compared with untreated plus vehicle.
which, depending on the dose, can even induce apoptosis in endothelial cells. Interleukin-1 is mostly known as an angiogenesis stimulator, but some reports identified inhibitory activities as well (36).

The role of NF-κB in endothelial cells is poorly understood (37). We found previously that, in bovine endothelial cells, 16K hPRL induces caspase-dependent apoptosis by a mechanism that requires activation of NF-κB (30). Interestingly, endostatin and anginex have also been described to induce apoptosis in endothelial cells (12). We here showed that NF-κB is induced in human and mouse endothelial cells after exposure to any of these inhibitors. However, specific inhibition of NF-κB does not counteract anginex-induced inhibition of proliferation/viability but abrogate anginex-induced inhibition of sprouting. These results suggest that even if NF-κB activation is a common pathway activated by angiostatic agents, the role of NF-κB is agent-specific and/or cell-specific.

Even if BAY1170-82 has been shown previously to target specifically the NF-κB pathway at concentrations <20 μmol/L, we cannot rule out the possibility that this compound may have off-target effects in endothelial cells. Therefore, p65 silencing would be an optimal strategy to confirm our results. However, transfection of primary endothelial cells such as HUVEC is a challenging option. In our hands, optimized transfection protocols gave an efficiency rate of ~30%, indicating that the NF-κB pathway could be blocked in a maximum of 30% of the cells and therefore making the data interpretation difficult. In addition, the toxicity we observed following transfection was not negligible, as is not unusual with primary endothelial cells, suggesting that many stress-related pathways would be activated by the transfection process and confuse the results about the role of NF-κB in angiogenesis.
Very recently, NF-κB signaling has been found to regulate endothelial cell integrity and vascular homeostasis in vivo. Treatment of zebrafish embryos with NF-κB inhibitors provoked vascular leakage and altered vessel morphology (38). Tie2 promoter/enhancer-1ακBαS32A/S36A transgenic mice, in which the endothelial-specific Tie2 promoter drives the expression of a trans-dominant-negative mutant of NF-κB and therefore present an inhibition of endothelial NF-κB signaling, developed normally and displayed, in particular, a normal pattern of vascular development (39). However, inoculated tumors grew faster in these mice, and histologic analysis revealed a striking increase in tumor vascularization. The molecular mechanisms accounting for such increase are still unknown. Our data are in accordance with these observations and furthermore suggest that NF-κB activation is mainly involved in the spatial organization of endothelial cells such as sprouting.

The activation of NF-κB can also be connected with an indirect antitumor activity through reversal of endothelial unresponsiveness to inflammatory signals, a process called endothelial cell anergy. The latter is defined by us as the absence of leukocyte-binding adhesion molecules on endothelial cells and the unresponsiveness to inflammatory cytokines (40). This phenomenon occurs due to exposure of endothelial cells to angiogenic growth factors. NF-κB is best known for its regulation of proinflammatory genes. We have reported previously that angiogenic factors (e.g., bFGF) down-regulate endothelial adhesion molecules such as ICAM-1, vascular cell adhesion molecule-1, and E-selectin through NF-κB inhibition (41). In addition, tumor endothelial cells displayed a reduced expression of adhesion molecules compared with that of normal endothelial cells, accounting for the observation of a reduced number of infiltrated leukocytes within the tumor (42). Accordingly, we found in the current study that NF-κB activation by angiostatic agents is correlated with ICAM-1 up-regulation at the endothelial cell surface. This expression further results in an enhanced NF-κB-dependent leukocyte-endothelial cell interaction. Together, these data show that anginex, and possibly other angiogenesis inhibitors as well, overcome endothelial cell anergy by activation of the NF-κB pathway. This activity may be shared with the abovementioned proinflammatory cytokines that can have intrinsic antiangiogenesis activities as well (34–36). Therefore, activation of NF-κB in endothelial cells resulting in stimulation of antitumor immunity can clearly result in an antitumor outcome.

The fact that different biochemically and biologically unrelated angiostatic agents activate the NF-κB pathway is intriguing. Recently, galectin-1 has been identified as the anginex receptor at the surface of endothelial cells (43). The importance of gal-1 in angiogenesis has been further illustrated in the zebrafish model, where expression knockdown results in impaired vascular guidance and growth of dysfunctional vessels. However, thus far, no direct evidence has linked galectin-1 and the NF-κB pathway. Endostatin may exert its antiangiogenic effect through its binding to αvβ3 integrin or to binding to glypican (44, 45). However, it is not obvious which endostatin receptor conveys the effective antiangiogenic signals. Our data showing that NF-κB pathway is activated by different angiostatic agents already after 1 h of treatment suggest that a common receptor/coreceptor exist at the surface of endothelial cells. Further studies will be required to decipher the pathway upstream NF-κB activation up to the receptor.

We report for the first time that activation of NF-κB is a common pathway used by multiple and biochemically unrelated angiostatic agents. NF-κB activation has been observed both in vitro and in vivo in tumor endothelial cells. Furthermore, we showed that NF-κB inhibition impairs the ability of angiostatic agents to block sprouting of endothelial cells and to overcome endothelial cell anergy. This is of special interest because, in tumor cells, NF-κB activation has been associated to inhibition of apoptosis. Therefore, currently novel treatment strategies are being developed based on inhibition of NF-κB. Although this would cause an antitumor effect in tumor cells, it is suggested to coincide with proangiogenic activity. Based on our results, the antitumor activity of angiostatic agents is linked to activation of NF-κB in endothelial cells. This activation could result in stimulation of antitumor immunity and in inhibition of angiogenesis. It is attractive to speculate that optimal therapeutic results could be achieved by either (or both) targeting inhibitors of NF-κB to tumor cells and/or by specific activation of NF-κB in endothelial cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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