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Dual functional roles of Tie-2/angiopoietin in TNF-α-mediated angiogenesis

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Chen, Jian-Xiong, Ying Chen, Laura DeBusk, Wenyu Lin, and Pengnain Charles Lin. Dual functional roles of Tie-2/angiopoietin in TNF-α-mediated angiogenesis. *Am J Physiol Heart Circ Physiol* 287: H187–H195, 2004; 10.1152/ajpheart.01058.2003.—Inflammation and angiogenesis are associated with pathological disorders. TNF-α is a major inflammatory cytokine that also regulates angiogenesis. TNF-α has been shown to regulate Tie-2 and angiopoietin (Ang) expression, but the functional significance is less clear. In this study, we showed that TNF-α induced a weak angiogenic response in a mouse cornea assay. Systemic overexpression of Ang-1 or Ang-2 dramatically increased corneal angiogenesis induced by TNF-α. In the absence of TNF-α, neither Ang-1 nor Ang-2 promoted corneal angiogenesis. Low doses (0–25 ng/ml) of TNF-α increased vascular branch formation of cultured endothelial cells. Overexpression of Ang-1 or Ang-2 enhanced the effects of TNF-α. These data suggest that Tie-2 signaling synergistically amplifies and participates in TNF-α-mediated angiogenesis. In addition, high doses (≥50 ng/ml) of TNF-α induced apoptosis in endothelial cells, but addition of Ang-1 or Ang-2 significantly reduced cell death. Enhanced endothelial cell survival was correlated with Akt phosphorylation. Collectively, our data reveal dual functional roles of Tie-2: low doses enhance TNF-α-induced angiogenesis, and high doses attenuate TNF-α-induced cell death. The study provides evidence supporting a role for Tie-2 in inflammatory angiogenesis.

inflammation; cell death

ONE OF THE MAJOR DIFFERENCES between physiological and pathological angiogenesis is the inflammation that accompanies pathological angiogenesis. Tissue injury induces inflammation, and inflammation triggers angiogenesis and initiates tissue growth and repair. A study of the molecular mechanism of pathological angiogenesis offers great potential in understanding the disease mechanisms as well as the development of therapeutic interventions. TNF-α is a major inflammatory cytokine. TNF-α also regulates angiogenesis (23). However, studies of the angiogenic properties of TNF-α yield contradictory results. TNF-α induces angiogenesis in vivo and stimulates endothelial cell migration in vitro (8, 21, 23), but TNF-α inhibits the action of mitogens such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF) in endothelial cell growth in vitro (8, 12, 33). Therefore, it has been suggested that the angiogenic properties of this cytokine might be mediated through various secondary angiogenic factors, such as platelet-derived growth factor, VEGF, IL-8, and basic fibroblast growth factor (29, 31, 45).

Tie-2 is a tyrosine kinase receptor that is predominantly expressed in endothelium and required for vascular development (7, 35). We have demonstrated that Tie-2 signaling plays a critical role in tumor angiogenesis. Blocking Tie-2 activation by using soluble Tie-2 (ExTek) as a Tie-2 inhibitor significantly inhibited tumor angiogenesis and tumor growth (24, 25). Tie-2 function has been linked to various pathological disorders, including tumor angiogenesis (14, 20, 39), retina neovascularization (13), and arthritis (38). Recently, we showed that Tie-2 function is required for TNF-α-induced corneal angiogenesis and inflammatory-mediated angiogenesis in arthritis (6).

Multiple ligands for Tie-2 have been identified and named angiopoietins (Ang) (4, 5, 27, 42) and have been detected in a variety of diseases (2, 15, 22, 30, 34, 37, 40). Ang-1 induces tyrosine phosphorylation of Tie-2 on endothelial cells (4), stabilizes vascular branch networks (16), and promotes endothelial cell survival against serum starvation (16). Ang-1 enhances endothelial cell survival through the phosphatidylinositol 3-kinase-Akt pathway (32). In contrast, Ang-2 has been considered a natural antagonist ligand of Tie-2 (27). In contrast to Ang-1, Ang-2 does not stimulate Tie-2 phosphorylation in endothelial cells (27). However, recent findings show that the action of Ang-2 as a Tie-2 agonist or antagonist is context dependent (10, 17, 41). At high concentration, Ang-2 activated the Tie-2 receptor in endothelial cells (17), revealing a more complex role of Ang-2 in angiogenesis. Neither Ang-1 nor Ang-2 directly promotes neovascularization in vivo in a corneal assay, but Ang-1 and Ang-2 enhance VEGF-induced angiogenesis (1).

TNF-α induces Tie-2 and Ang-2 expression in endothelial cells as well as Ang-1 expression in cultured human synoviocytes (18, 36, 44). However, the functional significance of Tie-2/Ang signaling in TNF-α-induced angiogenesis is not clear. In this study, we showed that Tie-2/Ang signaling synergistically amplified angiogenesis induced by low doses (0–25 ng/ml) of TNF-α. In addition, overexpression of Ang-1 or Ang-2 attenuated endothelial cell apoptosis induced by high doses (≥50 ng/ml) of TNF-α, presumably, through the Akt pathway. These results provide evidence demonstrating dual functional roles of Tie-2/Ang signaling in inflammatory angiogenesis and vascular survival and maintenance.

MATERIALS AND METHODS

Cell culture and reagents. Human umbilical vein endothelial cells (HUVECs) were purchased from the National Cancer Institute. HUVECs were cultured on gelatin-coated tissue culture dishes and kept in endothelial cell growth medium (EGM; Clonetics). HUVECs at *passages 3–7* were used in this study. Recombinant Ang-1* and Ang-2 proteins and adenoviral vectors directing the expression of Ang-1* (AdAng-1) and Ang-2 (AdAng-2) were provided by Dr. George Yancopoulos (Regeneron). Ang-1* is a slightly modified

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version of Ang-1 that is easier to express and purify (19). An adenoviral vector directing the expression of a green fluorescent protein (AdGFP) was used as a control vector. Viral vectors were propagated in 293 cells and purified in a CsCl column as described elsewhere (24). TNF-α was purchased from BD PharMingen.

Mouse cornea neovascularization assay. Gender- and age-matched BALB/c mice (8 wk old) were used for all experiments. The mice were housed in pathogen-free units at the Vanderbilt University School of Medicine in compliance with Institutional Animal Care and Use Committee regulations. The animal studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. The assay was performed as described previously (25). Briefly, a Hydron-sucralfate pellet containing 50 ng of TNF-α was surgically implanted into a cornea micropocket 0.5–1 mm from the limbus. The mice were randomly divided into groups and injected intravenously with AdAng-1, AdAng-2, AdExTek, or AdGFP at 1×10^9 plaque-forming units/mouse. Plasma was collected 2 days after viral injection for the examination of Ang-1 and Ang-2 expression in the circulation. Corneas were monitored daily for ingrowth of new microvessels from the limbus toward the pellet. Neovascularization could be seen 4 days after implantation, and continued extension into the pellet was observed for up to 10 days. At *day 7*, mice were perfused intraventricularly with india ink solution. The eyes were enucleated and fixed. The corneas were excised and placed on glass slides and examined by a microscope connected to a digital camera. Images were analyzed using NIH Image 1.61 software to determine the circumference area of neovascularization and the length of the vessels as indexes of corneal angiogenesis.

Western blot analysis for Ang-1 and Ang-2 expression. Adenoviral vector-mediated Ang-1 and Ang-2 expression in the circulation of mice was determined by Western blot analysis. Briefly, a small amount of blood was collected from the tail vein into a heparinized microcapillary branch 2 days after the viral injection. Plasma was recovered after brief centrifugation to remove blood cells, analyzed by SDS-PAGE, and then transferred to a nitrocellulose membrane. After the membrane was blocked, it was incubated with an anti-Ang-1 or

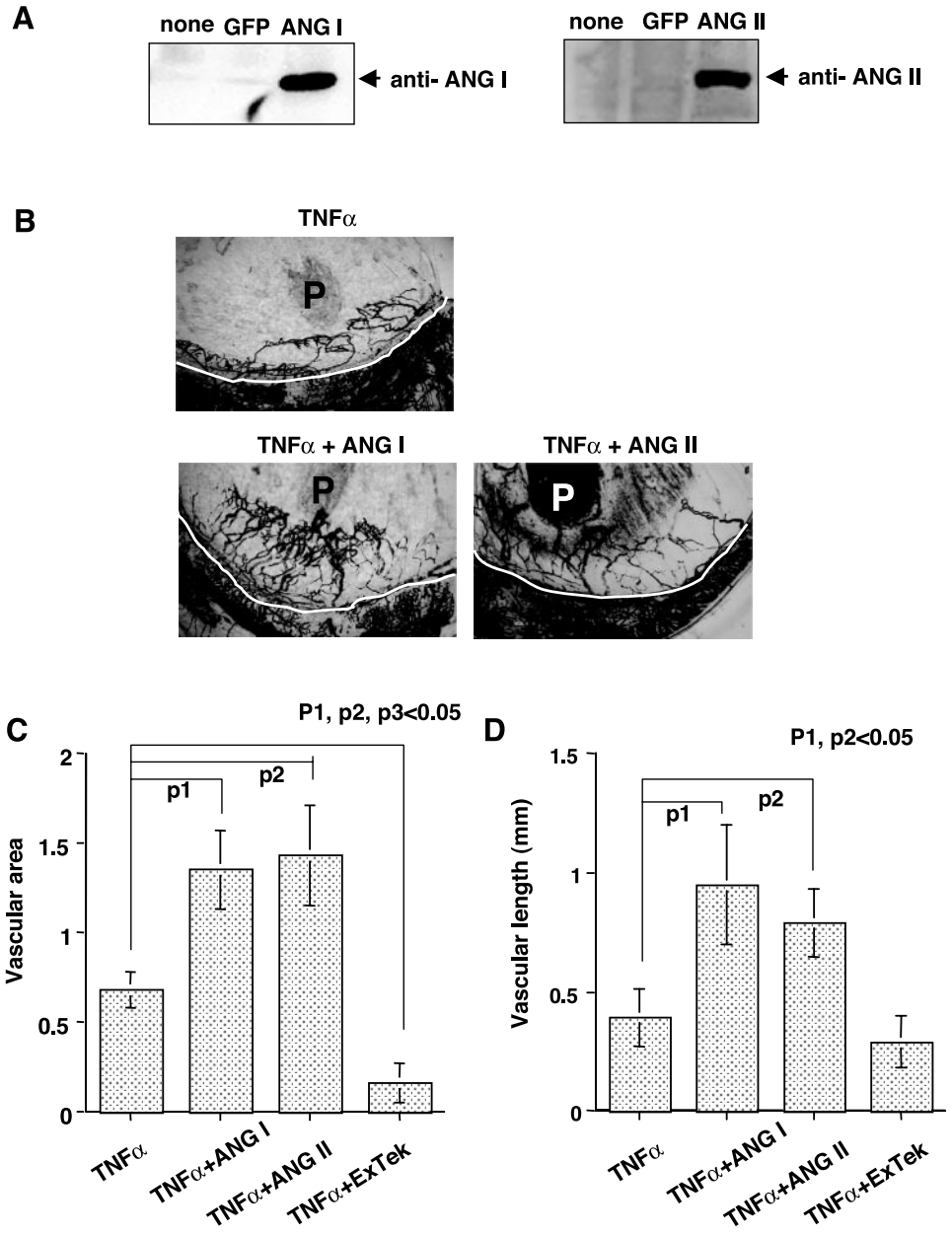

Fig. 1. Overexpression of angiopoietin (Ang)-1 or Ang-2 promotes TNF-α-induced corneal angiogenesis in vivo. TNF-α (50 ng/pellet) was implanted in a surgically created micropocket in mouse cornea. On the following day, animals received an intravenous injection of adenoviral vectors directing expression of Ang-1 (AdAng-1), Ang-2 (AdAng-2), ExTek (AdExTek), or control green fluorescent protein (AdGFP). Animal plasma was collected 2 days after viral injection. Normal animal plasma without viral injection was used as control. Plasma was analyzed in a Western blot and probed with an anti-Ang-1 or anti-Ang-2 antibody (*A*). At 7 days after pellet (P) implantation, animals were perfused with India ink, and the cornea was fixed and excised to reveal the pattern of vascular growth (*B*). Vascular area measured by the area covered by neovasculature (*C*) and vascular length measured by length of vessels from the limbus to the tip of the sprouts (*D*) were calculated using NIH Image 1.61 software. Five mice were used in each group. A two-tailed Student's *t*-test was used to analyze statistical differences (*C* and *D*).

anti-Ang-2 antibody (Santa Cruz Biotechnology) and then incubated with a horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using an enhanced chemiluminescence Western blotting analysis system (Perkin Elmer).

Viral infection of HUVECs. HUVECs were infected with five multiplicities of infectious particles of AdAng-1, AdAng-2, or control AdGFP. At 24 h after viral infection, the cells were trypsinized, counted, and prepared for further experiments. Transfection efficiency was determined by green fluorescent protein expression in cells infected with AdGFP.

Vascular branch formation assay. HUVECs infected with adenoviral vectors were trypsinized, counted, placed in 24-well tissue culture plates (2.5×10^4 cells/well), and maintained in endothelial basal medium (EBM; Clonetics). Cells were treated with TNF-α at various doses or vehicle alone as a control for 3 h and then covered with 300 μl of ECM gel (Sigma). The cells were kept at 37°C for 1 h to allow the gel to solidify; then EBM or EBM containing different concentrations of TNF-α was added into each well, and the cells were returned to 37°C. The cells were photographed under a phase-contrast microscope. The number of vascular branches was evaluated 48–72 h after the cells were plated by counting eight randomly chosen fields from triplicate wells. Vascular branches were defined as individual branches and a tube up to its branching point.

Detection of endothelial cell apoptosis. AdGFP-, AdAng-1-, or AdAng-2-infected HUVECs were plated onto 12-well plates (2.5×10^4 cells/well) and incubated in EGM for 24 h. The cells were then treated with vehicle as a control or with TNF-α at 50 μg/ml for another 24 h. To quantify viable cells, total cells, including adhering cells and floating cells, were collected from each group. The cells were stained with trypan blue, and live cells were counted under a microscope. In addition, genomic DNA fragmentation was determined by the TdT-mediated dUTP nick end labeling assay following the manufacturer's instruction (Intergen).

Akt phosphorylation assay. HUVECs were serum starved in EBM for 4 h and then infected with AdAng-1, AdAng-2, or control AdGFP. Cells were harvested at 24 h after viral infection. In the time-course

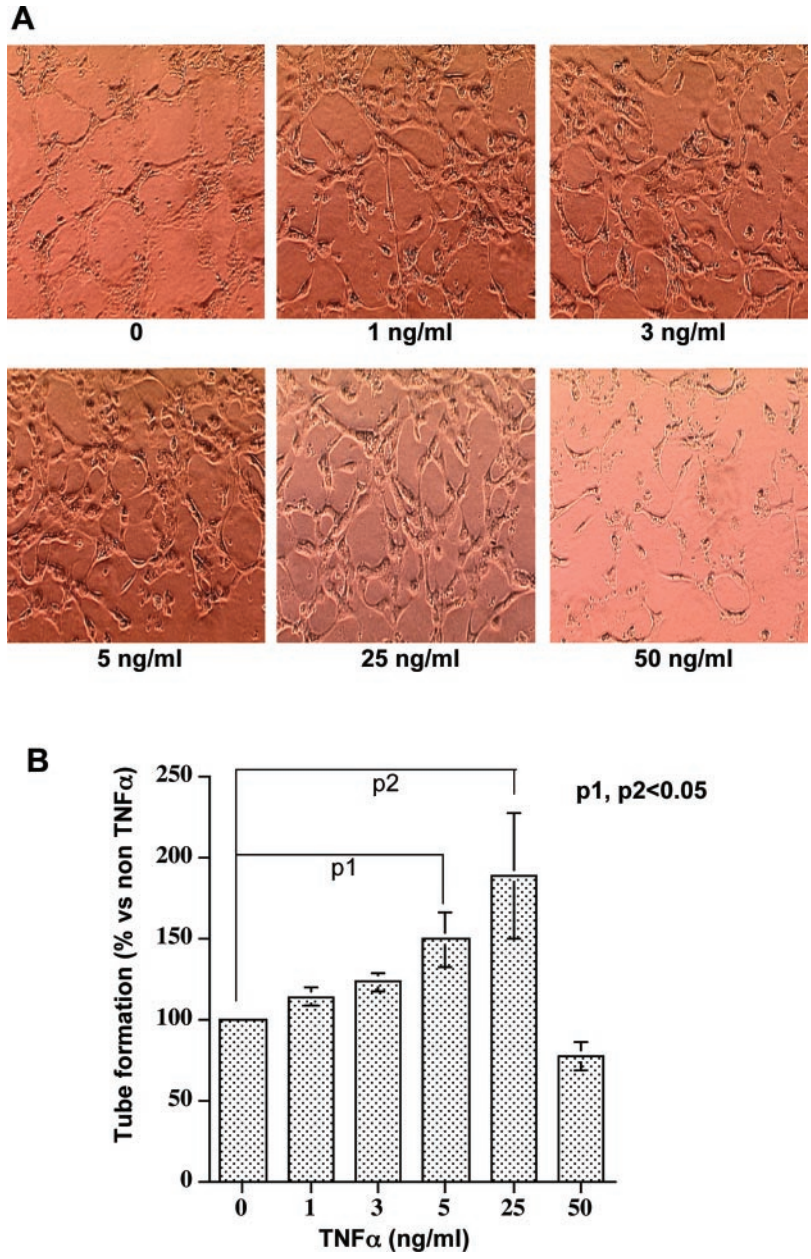

Fig. 2. Low doses of TNF-α induce vascular branch formation in a 3-dimensional gel. Cultured human umbilical vein endothelial cells (HUVECs) were treated with TNF-α at 0–50 ng/ml for 3 h and then embedded with ECM gel and maintained in serum-free endothelial basal medium (EBM). Increasing amounts of TNF-α were added to the culture medium. Micrographs were obtained 48–72 h after cells were covered with ECM gel under an inverted microscope (*A*). Vascular branch formation was measured by counting the number of branch structures in 8 randomly selected fields (×100) with an inverted microscope. Vascular branch formation index was calculated as the ratio of each TNF-α-treated group to the nontreated group (*B*). Each experiment was done in triplicate, and experiments were repeated twice. A two-tailed Student's *t*-test was used to analyze statistical differences between control group and each TNF-α-treated group.

experiment, HUVECs were stimulated with recombinant Ang-1* (200 ng/ml) or Ang-2 (200 ng/ml) protein for up to 24 h. Cells were then lysed in RIPA buffer + protease inhibitors and sodium vanadate. Cellular proteins were collected, and the protein content was measured using a bicinchoninic acid protein assay kit (Bio-Rad). Proteins (20 μg/sample) were separated on an SDS-10% polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed with a phosphorylated Akt (Ser^{473}) antibody (Cell Signaling) for 1 h at room temperature. The membrane was then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Promega). The membrane was developed using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech). The same membranes were stripped and reblotted with an anti-Akt antibody that detects the total Akt (Cell Signaling).

Statistics. Values are means ± SE. A two-tailed Student's *t*-test was used to analyze statistical differences between control and treated groups. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Ang-1 and Ang-2 significantly enhance TNF-α-induced cornea angiogenesis in vivo. TNF-α has been shown to induce cornea angiogenesis in vivo (23). Our previous studies show that Tie-2 function is required for TNF-α-mediated angiogenesis in vivo (6). Here we used a mouse corneal angiogenesis assay to examine whether angiopoietins affect TNF-α-mediated angiogenesis. A small hydron pellet containing TNF-α (50 $ng \cdot pellet^{-1} \cdot eye^{-1}$) was implanted in a surgically created micropocket in a mouse cornea. On the following day, the mice were randomly divided into groups and received an intravenous injection of adenoviral vectors directing the gene expression of interest. High levels of Ang-1 and Ang-2 were detected in the circulation 2 days after viral injection (Fig. 1*A*) compared with control virally infected mice and normal mice without viral injection, and gene expression lasted for 1 wk (data not shown). Corneal angiogenesis was evaluated by measuring the vascular length (from the limbus to the tip of the sprouts) and the vascular area of ingrowth vessels 7 days after surgery. TNF-α alone induced a weak corneal angiogenic response, and a few vessels extended from the limbus toward the pellet (Fig. 1*B*). In contrast, overexpression of Ang-1 or Ang-2 systemically markedly increased corneal angiogenesis induced by TNF-α (Fig. 1*B*). Neither control pellets containing vehicle nor administration of AdAng-1 or AdAng-2 intravenously in the absence of TNF-α induced cornea neovascularization (data not shown). Reciprocally, we have shown that blocking Tie-2 function by overexpressing a soluble Tie-2 receptor, ExTek, inhibited TNF-α-induced corneal angiogenesis (6). After quantification of the neovascular area and the vascular length, we observed that overexpression of Ang-1 or Ang-2 significantly increased the vascular area and vascular length by twofold (Fig. 1, *C* and *D*; $P < 0.05$). The mean value of the vascular area index is 0.697, 1.364, 1.454, and 0.167 for groups treated with TNF-α, TNF-α + Ang-1, TNF-α +

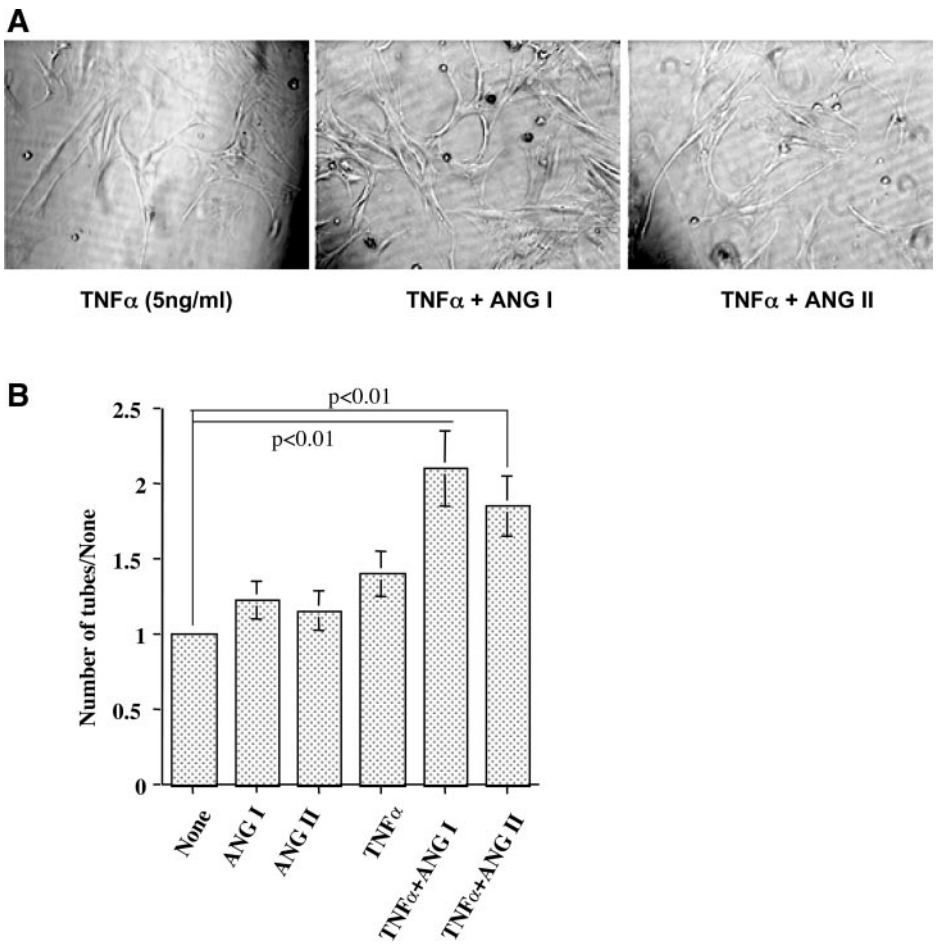

Fig. 3. Overexpression of Ang-1 or Ang-2 enhances TNF-α-induced vascular branch formation in vitro. Cultured HUVECs were infected with AdAng-1, AdAng-2, or control AdGFP for 24 h and then seeded onto a 24-well plate. Cells were treated with TNF-α at 5 ng/ml for 3 h and then covered with ECM gel and maintained in EBM. TNF-α at 5 ng/ml was added to the culture medium. Micrographs were obtained 48–72 h after cells were covered with ECM gel under an inverted microscope (*A*). Vascular branch formation was measured by counting the number of branch structures in 8 randomly selected fields (×100). Vascular branch formation index was calculated as the ratio of each treatment group to control group (*B*). Each experiment was done in triplicate, and experiments were repeated twice. A two-tailed Student's *t*-test was used to analyze statistical differences between group treated with TNF-α and group treated with TNF-α + Ang-1 or Ang-2.

Ang-2, and TNF-α + ExTek, respectively (Fig. 1*C*). The mean vascular length is 0.38, 0.955, 0.795, and 0.284 mm for groups treated with TNF-α, TNF-α + Ang-1, TNF-α + Ang-2, and TNF-α + ExTek, respectively (Fig. 1*D*). These findings show that Tie-2/Ang signaling contributes to TNF-α-induced angiogenesis in vivo and suggest that Tie-2 signaling plays a role in inflammation-related pathological angiogenesis.

Ang-1 and Ang-2 promote TNF-α-induced capillary branch formation in vitro. To further explore these findings, we examined whether Tie-2/Ang signaling contributed to TNF-α-induced angiogenesis in vitro. We analyzed the ability of TNF-α to promote endothelial cells to form vascular branches in a three-dimensional ECM gel (Sigma). A monolayer of cells was embedded beneath ECM gel and then overlaid with EBM. Various quantities of TNF-α were added to the culture medium in each well. Capillary branch formation was analyzed 48–72 h later. The morphology of the capillary branch was recorded using an inverted Olympus phase-contrast microscope connected to a digital camera. The number of capillary branches was counted at eight randomly selected fields (×100). At a lower dose range (0–25 ng/ml), TNF-α induced vascular branching in a dose-dependent manner (Fig. 2). However, at high doses (≥50 ng/ml) of TNF-α, we observed a dramatic decrease in vascular branching (Fig. 2). The cells showed rounding up, and apoptosis was observed.

Next, we examined the role of Tie-2 signaling in TNF-α-induced vascular branching by overexpression of Ang-1 or Ang-2. HUVECs were infected with AdAng-1, AdAng-2, or control AdGFP. The cells were then used to perform the vascular branch assay in ECM gel. TNF-α was added to the culture medium at 5 ng/ml. The morphology of the vascular branches was recorded and counted at eight randomly selected fields. As shown earlier, TNF-α promoted vascular branch formation. Overexpression of Ang-1 or Ang-2 slightly increased vascular branch formation in the absence of TNF-α compared with the control group. Interestingly, overexpression of Ang-1 or Ang-2 significantly increased branch formation in the presence of TNF-α (Fig. 3). There seemed to be slightly more branch structures in the Ang-1- than in the Ang-2-treated group (Fig. 3*A*). These results are in accordance with the in vivo data that Tie-2 signaling enhances TNF-α-induced vascular branch formation in vitro. They also support our findings that Tie-2 signaling modulates TNF-α-mediated angiogenesis

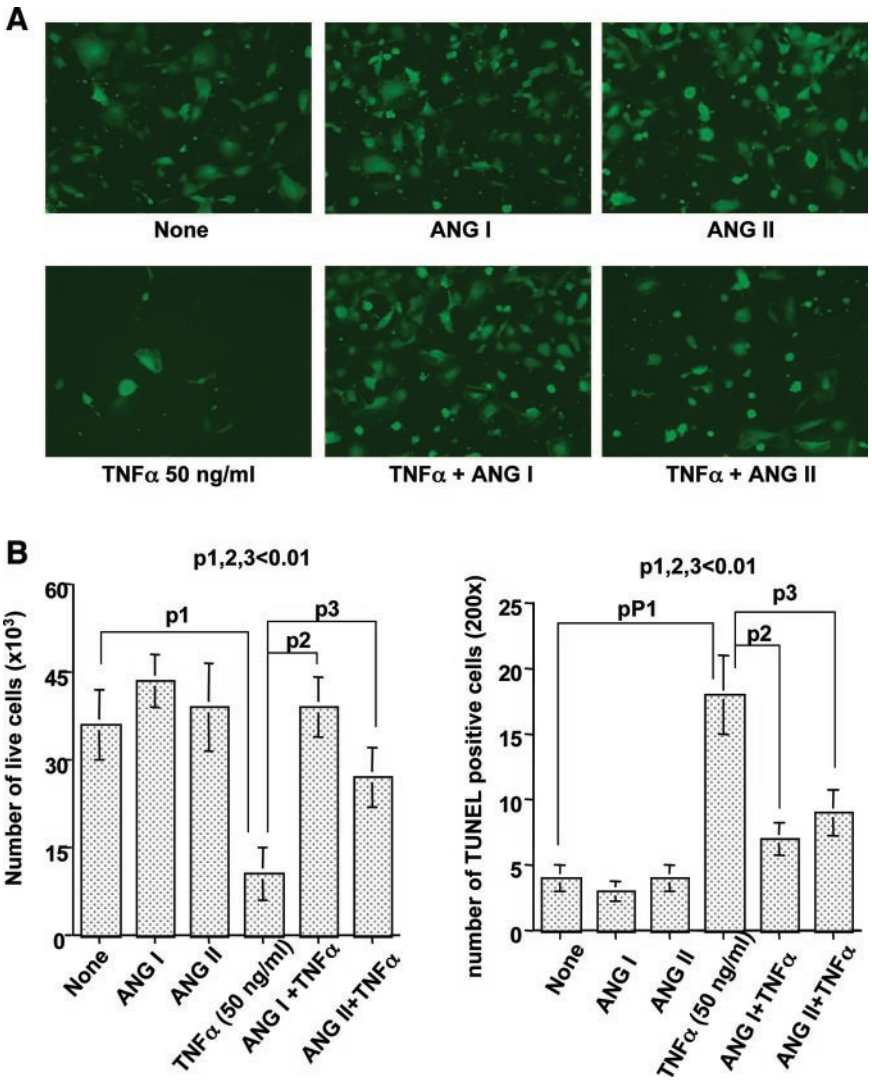

Fig. 4. Overexpression of Ang-1 or Ang-2 attenuated endothelial cell death induced by high doses of TNF-α. Cultured HUVECs were infected with AdAng-1, AdAng-2, or control AdGFP for 24 h. Cells were maintained in EBM. One set of cells was treated with TNF-α at 50 ng/ml, and another set was left without treatment. Micrographs were taken 24 h after TNF-α treatment. Live cells can be easily visualized, because infected cells also express GFP (*A*). Adhering cells and floating cells from each group were collected. Cells were incubated with trypan blue. Live cells were counted under an inverted microscope (*B*). Genomic DNA fragmentation was determined by TdT-mediated dUTP nick end labeling (TUNEL) assay 16 h after treatment with TNF-α at 50 ng/ml. Apoptotic cells were counted under a high-power field (×200) in 10 randomly selected fields (*C*). Experiment was done in triplicate, and experiments were repeated twice. A two-tailed Student's *t*-test was used to analyze statistical differences between each group.

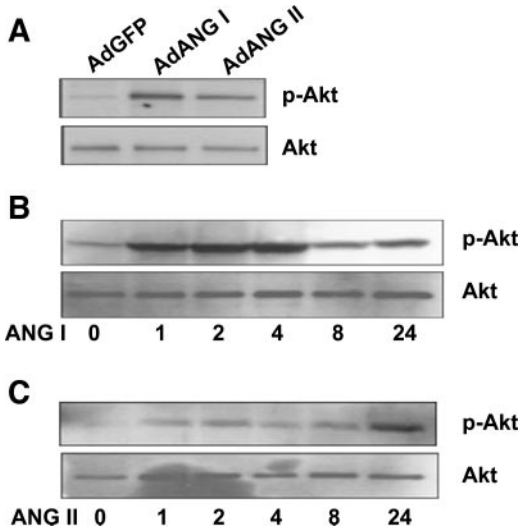

Fig. 5. Stimulation of endothelial cells with Ang-1 or Ang-2 activates Akt in the presence of TNF-α. Cultured HUVECs were infected with AdAng-1, AdAng-2, or control AdGFP. Ang-1 or Ang-2 protein was continuously produced and secreted into the medium. Cells were constantly exposed to the ligands. At 24 h after viral infection, cells were maintained in EBM and treated with TNF-α at 50 ng/ml for another 12 h; then cells were lysed using RIPA buffer. Equal amounts of protein were analyzed by a Western blot and probed with an anti-phospho-Akt antibody. The same membrane was stripped and reprobed with an anti-Akt antibody (*A*). HUVECs were also stimulated with recombinant Ang-1* (*B*) or Ang-2 (*C*) protein at 200 ng/ml for up to 24 h in EBM. Cells were harvested at different times after stimulation. Cell lysates were analyzed by Western blot and probed with an anti-phospho-Akt antibody. The same membrane was stripped and reprobed with an anti-Akt antibody. Experiments were repeated twice. p-Akt, phosphorylated Akt.

and may play a role in inflammatory-related pathological angiogenesis in vivo.

Ang-1 and Ang-2 attenuate endothelial cell apoptosis induced by TNF-α and potentially through the Akt pathway. It has been reported that TNF-α induces apoptosis (26). In the capillary branch formation experiment, we observed that high doses of TNF-α dramatically reduced vascular branch formation, and the cells appeared unhealthy. Our observations suggested that the reduction of vascular branch formation might be due to cell death. Tie-2 function has been linked to endothelial cell survival and vascular maintenance. Thus we investigated whether Tie-2 activation could protect endothelial cells from TNF-α-induced apoptosis. HUVECs were infected with AdAng-1, AdAng-2, or a control AdGFP viral vector. The cells were then treated with TNF-α at 50 ng/ml. The morphology of the cells was recorded using an inverted phase-contrast microscope connected to a digital camera. A high TNF-α concentration induced HUVEC apoptosis, and few live cells were attached to the cell culture dish compared with the vehicle-treated control group. Overexpression of Ang-1 or Ang-2 attenuated cell death induced by a high dose TNF-α (Fig. 4*A*). Adhering and floating cells were collected and stained with trypan blue. Viable cells from each group were counted. The data confirmed that TNF-α at 50 ng/ml enhanced endothelial cell death, and addition of Ang-1 or Ang-2 significantly inhibited TNF-α-induced cell death (Fig. 4*B*). Furthermore, overexpression of Ang-1 yielded stronger effects on HUVECs than overexpression of Ang-2 (Fig. 4*B*). To further confirm our finding, TdT-mediated dUTP nick end labeling assay was used to measure cell apoptosis on cultured HUVECs 16 h after treatment with TNF-α at 50 ng/ml. The data showed that high doses of TNF-α induced apoptosis in HUVECs that could be significantly reversed by the addition of Ang-1 or Ang-2 (Fig. 4*C*). These results reveal that Tie-2 signaling protects endothelial cells from apoptosis induced by a high dose of TNF-α. Tie-2 signaling may play a role in vascular survival/maintenance and in protecting the vasculature from a pathological environment.

Akt plays a critical role in cell survival. Akt has been shown to regulate Tie-2-mediated endothelial cell survival against growth factor depletion (16, 32). We examined the Akt phosphorylation levels on stimulation with Ang-1 and Ang-2 in the presence of TNF-α. HUVECs were infected with different viral vectors for 24 h and then treated with TNF-α at 50 ng/ml for another 12 h in EBM. Akt phosphorylation was examined by Western blot and reacted with an anti-phospho-Akt antibody. Long-term exposure of HUVECs to Ang-1 or Ang-2 by infecting the cells induced significantly higher Akt phosphorylation than the control (Fig. 5*A*). To confirm this finding, we stimulated HUVECs with recombinant Ang-1* or Ang-2 protein at 200 ng/ml. The cells were harvested at different times, and Akt activation was analyzed by Western blot analysis. Stimulation with recombinant Ang-1 protein induced an acute Akt activation as expected. The phosphorylated Akt levels significantly increased 1 h after Ang-1 protein stimulation and lasted for 4 h; then they decreased to baseline (Fig. 5*B*). In contrast, Ang-2 stimulation led to a delayed Akt activation. There was a minimal change in the phosphorylated Akt level during the first 8 h after stimulation, but a strong phosphorylation of Akt was detected 24 h after stimulation (Fig. 5*C*). Collectively, the data show that Tie-2 signaling attenuates TNF-α-induced endothelial cell death, which correlates with Akt activation.

DISCUSSION

Our study shows that Tie-2/Ang signaling modulates TNF-α-induced angiogenesis. Low doses (0–25 ng/ml) of TNF-α

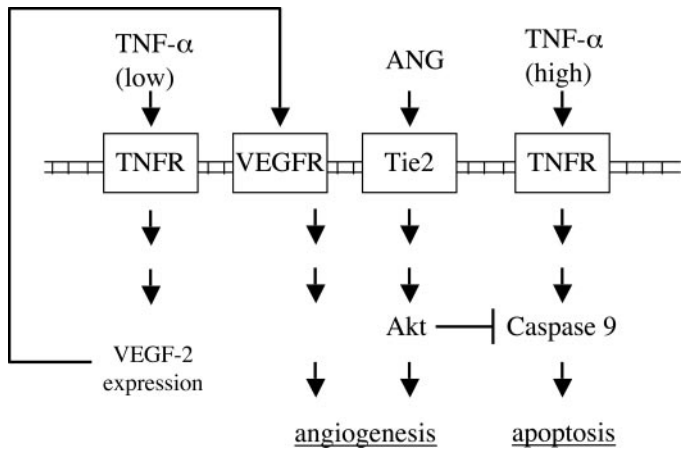

Fig. 6. Potential mechanisms of dual functional roles of Tie-2/Ang in TNF-α-mediated angiogenesis. Low doses of TNF-α induce expression of vascular endothelial growth factor (VEGF) receptor (VEGFR)-2 and collaboratively work with Tie-2/Ang signaling and promote angiogenesis. High doses of TNF-α induce apoptosis via activation of caspase-9, which could be inhibited by Akt activation induced by Tie-2/Ang signaling. TNFR, TNF-α receptor.

induced a weak angiogenesis in vivo and in vitro. The angiogenic function of TNF-α could be significantly enhanced by overexpression of Ang-1 or Ang-2. Reciprocally, our previous findings show that blocking Tie-2 function inhibits the TNF-α-induced angiogenesis (6). On the contrary, high levels (≥50 ng/ml) of TNF-α induced endothelial cell death. Overexpression of Ang-1 or Ang-2 attenuated the cell death induced by TNF-α. The enhanced endothelial cell survival was correlated with Akt phosphorylation, suggesting that Tie-2/Ang-induced endothelial cell survival was mediated through the Akt pathway. The data reveal dual functional roles of TNF-α in regulating vascular formation and vascular survival and dual roles of Tie-2/Ang in mediating TNF-α-induced angiogenesis.

Inflammation-related angiogenesis is a hallmark of pathological angiogenesis that plays essential roles in disease development (43). TNF-α is a major inflammatory cytokine and also regulates angiogenesis (23). However, contradictory findings have been reported regarding the angiogenic properties of TNF-α (8, 12, 33). It has been suggested that the angiogenic properties of TNF-α may be mediated through various secondary angiogenic factors (29, 31, 45). Here, we show that Tie-2/Ang signaling modulates TNF-α-induced angiogenesis. The angiogenic properties of angiopoietins are more subtle than those of VEGF. Neither Ang-1 nor Ang-2 alone induces adult angiogenesis in a corneal assay, but Ang-1 and Ang-2 enhance VEGF-mediated corneal angiogenesis in vivo (1). In the present study, we observed similar findings that Ang-1 or Ang-2 alone failed to induce corneal angiogenesis. Ang-1 and Ang-2 significantly enhanced the angiogenic effects of TNF-α. A previous study (11) and our data (unpublished data) showed that low doses of TNF-α also increase the expression of VEGF receptor-2 in endothelial cells, which could explain the synergistic effects of angiopoietins and TNF-α in regulating angiogenesis (Fig. 6). Investigating the molecular mechanisms of inflammation-induced angiogenesis holds tremendous potential for understanding the mechanisms of disease progression as well as developing better and more specific inhibitors for the treatment.

Tie-2 regulates endothelium survival through the phosphatidylinositol 3-kinase-Akt pathway (9, 16). Akt belongs to a class of serine/threonine protein kinases that play a critical role in endothelium survival. Our data show that Akt is a major endothelial cell survival mediator downstream of the Tie-2 pathway against serum starvation (unpublished data). TNF-α induces cell death through the activation of caspase-9. Akt phosphorylates caspase-9 and suppresses its proapoptotic function (3). In this study, we observed that Tie-2 activation protected endothelial cells from TNF-α-induced cell death, and the cell survival was correlated with Akt activation. Therefore, Akt activation could account for the potent survival effect mediated by Tie-2/Ang signaling (Fig. 6).

Among the ligands for Tie-2 (4, 27, 42), Ang-1 induces Tie-2 phosphorylation (4) and Ang-2 has been shown to block Ang-1-induced Tie-2 phosphorylation in endothelial cells (27). Ang-2 has been considered an antagonist ligand. However, the present study shows that not only Ang-1, but also Ang-2, promotes TNF-α-mediated angiogenesis. Ang-1 protects endothelial cells from the cytotoxicity of high levels of TNF-α as expected. Surprisingly, Ang-2 also protected cells from the cytotoxicity of TNF-α. We observed that sustained stimulation of endothelial cells with Ang-2 by use of adenoviral vector induced Akt phosphorylation. Interestingly, one-time treatment with recombinant Ang-2 protein produced a delayed activation of Akt with a peak time at ~24 h after stimulation compared with an acute Akt activation on Ang-1 exposure. These results provide evidence supporting a role for Ang-2 in pathological vascular formation and vascular survival. In vitro studies regarding Ang-2 as an agonist or antagonist yield conflicting results. The group that cloned the gene also reported that Ang-2 induced Tie-2 phosphorylation when Tie-2 was expressed in fibroblast NIH 3T3 cells (27), suggesting that intracellular signaling may affect receptor activation. A higher dose of Ang-2 treatment was shown to activate Tie-2 and Akt in endothelial cells (17). Mochizuki and colleagues (28) recently reported that Ang-2 stimulated migration and tubelike structure formation through c-Fes and c-Fyn in endothelial cells. Clearly, there are more questions about Ang-2 that need to be addressed. Ang-2 may not simply act as an antagonist ligand. The biological function of Ang-2 may reflect the local environment and its concentration. It may depend on cell types and downstream signaling events. In the present study, it is not clear whether the delayed Akt activation is a direct response of Ang-2 or an indirect response via other factors. Current effort is focused on addressing these important questions and trying to understand the molecular mechanisms of Tie-2 signaling in TNF-α-mediated angiogenesis.

In conclusion, we have shown that TNF-α has dual functional roles in regulation of angiogenesis and vascular survival. At a low concentration, TNF-α induces angiogenesis. At a high concentration, TNF-α promotes endothelial cell death. We also observed dual functional roles of Tie-2/Ang signaling in TNF-α-mediated angiogenesis. Tie-2/Ang signaling enhances TNF-α mediated angiogenesis. Tie-2/Ang signaling also protects endothelial cells from cytotoxicity caused by a higher dose of TNF-α. The inflammatory environment contains a variety of cytokines and growth factors at high levels, which may present a pathogenic environment for the endothelium. Maintenance of a normal and functional vascular network is essential for disease development. Tie-2 signaling may protect pathological vasculature and promote disease progression.

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