Angiopoietin-1 Inhibits Vascular Permeability, Angiogenesis, and Growth of Hepatic Colon Cancer Tumors

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ABSTRACT

Angiopoietin (Ang)-1 and -2 are critical regulators of embryonic and postnatal neovascularization. Ang-1 activates the endothelial cell-specific tyrosine kinase receptor Tie-2, which in turn leads to enhanced endothelial cell survival and stabilization. The effects of Ang-1 on tumor angiogenesis remain controversial; although we have previously demonstrated that Ang-1 overexpression in colon cancer cells leads to a decrease in s.c. tumor growth, others have shown that Ang-1 may be proangiogenic. Few studies have addressed the role of the Angs in tumors growing in the organ of metastatic growth. We hypothesized that overexpression of Ang-1 may inhibit the growth of colon cancers growing in the liver by inhibition of angiogenesis. We also wanted to investigate the mechanisms by which Ang-1 affects angiogenesis in vivo. Human colon cancer cells (HT29) were stably transfected with an Ang-1 construct or an empty vector (pcDNA3) and injected directly into the livers of nude mice. After 37 days, livers were harvested and weighed, and tumor sizes were measured. In an additional experiment, to validate the paracrine effect of Ang-1, various mixtures of control cells and Ang-1-transfected cells were injected into mice, and tumor growth was assessed. Direct effects of recombinant Ang-1 on angiogenesis were studied with an in vivo Gelfoam angiogenesis assay. The impact of Ang-1 on vascular permeability was investigated using an intradermal Miles assay with conditioned media from transfected cells. Liver weights (P < 0.05), tumor volumes (P < 0.05), vessel counts (P < 0.01), and tumor cell proliferation (P < 0.01) in the Ang-1 group were significantly lower than those in the control (pcDNA3) group. Tumor vessels in the Ang-1 group developed a significantly higher degree of pericyte coverage (P < 0.02) than vessels in pcDNA3 tumors. In the cell mixture experiment, even as few as a 1:10 mixture of Ang-1-transfected cells/control cells resulted in a significant reduction of hepatic tumor volumes (P < 0.04). In the angiogenesis assay, vessel counts in Gelfoam implants were significantly decreased by the addition of Ang-1 (P < 0.01). Finally, conditioned medium from Ang-1-transfected cells decreased vascular permeability more than that from control cells (P < 0.05). Our results suggest that Ang-1 is an important regulator of angiogenesis and vascular permeability and that this effect may be secondary to increasing periendothelial support and vessel stabilization. Thus, Ang-1 could potentially serve as an antineoplastic or anti-permeability agent for patients with metastatic colorectal cancer.

INTRODUCTION

The process of angiogenesis is essential for tumor growth and metastases formation and has been associated with aggressive disease in human colorectal cancer (1–5). Proangiogenic factors such as VEGF3 promote EC proliferation, invasion, and angiogenesis. However, the activity of angiogenic factors is modulated by other factors that affect EC survival and attachment to surrounding structures. Recently, the Angs (Ang-1 to Ang-4) have been shown to be important mediators of angiogenesis by their regulation of EC survival in malignant and nonmalignant tissues (6). Ang-1 has been identified as a major activator of the tyrosine kinase receptor Tie-2 (7), resulting in a downstream activation of the phosphatidylinositol 3′-kinase/Akt survival pathway, thereby promoting EC survival (7). Ang-2 is the naturally occurring antagonist to Ang-1 and prevents Tie-2 activation; this effect leads to vessel destabilization, a necessary step in the initiation of angiogenesis by VEGF (8). Balanced and sequential expression of Angs and VEGF is required for successful angiogenesis (9, 10). Ang-1 has also been shown to override VEGF-mediated effects on vascular permeability [vessel leakage (11, 12)].

In human colorectal cancer, Angs seem to be expressed differently in tumors and nonmalignant tissues (13). In previous studies from our laboratory, Ang-2 was expressed ubiquitously in tumor epithelium of human colon cancer specimens, whereas expression of Ang-1 in tumor epithelium was rarely detected. This observation suggests that a net gain in Ang-2 activity over Ang-1 activity might be an initiating factor for tumor angiogenesis (14–16).

Although several investigators have shown that loss of Ang-1 activity may augment tumor angiogenesis, others have suggested that Ang-1 is proangiogenic (17–26). Thus, the effects of Angs on angiogenesis and tumor growth remain controversial. In a previous study, we demonstrated that imbalances in Ang expression significantly affected angiogenesis and tumor growth of s.c. implanted colon cancer cells [HT29 (27)]. In that study, Ang-1 overexpression inhibited angiogenesis and tumor growth of s.c. xenografts. However, the role of Ang-1 in tumor angiogenesis and growth at the site of metastatic tumor growth remains undefined. The influence of Ang-1 in the mediation of tumor vascular permeability and pericyte coverage is also poorly defined.

In this study, we hypothesized that overexpression of Ang-1 by tumor cells would impair angiogenesis and thereby inhibit tumor growth of human colon cancer cells transplanted into livers of nude mice (the liver is the most common site of colon cancer metastases). We also investigated the pure angiogenic effect of a novel recombinant Ang-1 (Ang-1 TFD) in an in vivo angiogenesis assay and investigated the role of Ang-1 in vascular permeability and pericyte coverage.

MATERIALS AND METHODS

Cell Culture. The human colon cancer cell line HT29 and HUVECs were purchased from the American Type Culture Collection (Manassas, VA). HT29 cells were maintained in MEM supplemented with 10% FBS, 2 units/ml penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD), a 2× vitamin solution (Life Technologies, Inc., Grand Island, NY), 1 mM sodium pyruvate, 2 mM l-glutamine, and nonessential amino acids and incubated in 5% CO2-95% air at 37°C, as described previously (27). HUVECs were

endothelial cell; TFD, tetra fibrinogen domain; OCT, optimum cutting temperature; PECAM, platelet/endothelial cell adhesion molecule; PCNA, proliferating cell nuclear antigen; α-SMA, α-smooth muscle actin; CM, conditioned medium.
cultured in MEM supplemented with 15% FBS and basic fibroblast growth factor as described previously (28).

Stable Transfection. The full-length cDNA for Ang-1 was obtained from Dr. T. Gilmer (GlaxoSmithKline, Research Triangle Park, NC). The construct was cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA) containing a hygromycin resistance gene. The vector containing Ang-1 or the empty pcDNA vector was transfected into HT29 cells with Lipofectin according to the manufacturer’s protocol (Boehringer Mannheim Co., Randburg, South Africa (27)). Cells were then grown in selective media (10% FBS-MEM containing 200 ng/ml hygromycin). Cell clones were subsequently screened by Northern blot analysis for an increase in Ang-1 mRNA expression relative to that in the pcDNA-transfected cells, as described previously (27). For in vitro experiments, HT29 cells that had been transfected with Ang-1 or pcDNA were harvested from subconfluent cultures by rinsing them with PBS and trypsinizing (0.25% trypsin and 0.02% EDTA) them for 3 min. Cells were washed in 10% FBS-MEM and counted. Cell viability was assayed by trypan blue exclusion, verifying that cell viability was >90% in all cell lines. Cells were then centrifuged and resuspended in HBSS for injection into mice.

Quantification of VEGF Protein in CM from Transfected Colon Cancer Cells. VEGF protein concentrations in conditioned media from Ang-1- or pcDNA-transfected HT29 cells were determined using an ELISA kit for human VEGF (Biosource International, Camarillo, CA). Cell culture supernatants (3 ml) from cells were collected after a 48-h incubation period [in 10% FBS-MEM (FBS does not contain detectable human VEGF)]. Supernatants were subsequently collected after centrifugation for 5 min at 350 × g. In parallel, cells in culture flasks were rinsed with PBS, trypsinized as described above, resuspended in 10% FBS-MEM, and counted for each cell line. VEGF ELISA of 10-fold-diluted conditioned media (due to excessive high VEGF levels) was performed according to the manufacturer’s protocol.

Animals. Eight-week-old male athymic nude mice or BALB/c mice (both obtained from the Animal Production Area of the National Cancer Institute and Development Center, Frederick, MD) were acclimated for 1–2 weeks while caged in groups of five. Mice were housed as described previously (29, 30) and fed a diet of animal chow and water ad libitum throughout the experiment. All experiments were approved by the Institutional Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center.

Colon Cancer Liver Tumor Model. To determine the effects of Ang-1 transfection on hepatic tumor growth of human colon cancer cells, Ang-1-transfected or pcDNA-transfected (control) cells (1 × 10^6 cells in 50-μl injection volume) were directly injected into the livers of athymic nude mice after they had been randomly assigned to one of the two groups (7–9 mice/group). Body weight was similar between the groups at the beginning of the experiment. Mice were observed daily, and all mice were killed (when three in a group showed decreased mobility or discomfort) by cervical dislocation followed by immersion with pentobarbital sodium (50 mg/ml). Body weights were measured, livers were excised, and liver weights and tumor diameters were subsequently determined. Tumor volumes were calculated with the equation width^2 × length × 0.5. Tumor size was then harvested and either placed in 10% formalin for paraffin embedding or snap-frozen in OCT solution (Miles Inc., Elkhart, IN) in preparation for subsequent immunohistochemical analyses.

To confirm the paracrine effect of Ang-1 on in vivo angiogenesis, Ang-1 and pcDNA cells were mixed at various ratios (100% Ang-1:0% pcDNA, 50% Ang-1:50% pcDNA, 10% Ang-1:90% pcDNA, and 0% Ang-1:100% pcDNA) and injected into the liver as described above. Mice were observed and sacrificed according to the criteria described above. Tumor-bearing livers were excised, their weights were determined, and tumor diameters were measured.

Gelfoam in Vivo Angiogenesis Assay. Effects of Ang-1 on angiogenesis were investigated in vivo using a syngeneic murine Red-tumor angiogenesis assay. Animals (five mice/group) received recombinant human Ang-1 [Ang-1 TFD (clustered Human Ang-1:50% pcDNA, 10% Ang-1:90% pcDNA, and 0% Ang-1:100% pcDNA) and injected into the liver as described above. Mice were observed and sacrificed according to the criteria described above. Tumor-bearing livers were excised, their weights were determined, and tumor diameters were measured.

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Angiogenesis Assay. Mice were sacrificed 14 days after the injection of tumor cells. Tumors were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E to study overall tissue structure. The numbers of PCNA-positive and PCNA-negative tumor cells were determined in 4 random fields/tumor (at ×100 magnification), and the percentage of PCNA-positive cells was then calculated.

Immunofluorescent Analyses of Pericyte-covered Tumor Vessels and Ang-1 Expression in Hepatic Tumors. To determine pericyte coverage of tumor vessels in pcDNA-transfected and Ang-1-transfected liver tumors, double staining for CD31 and α-SMA (Dako) was performed according to a modified protocol as described elsewhere (33). Frozen sections of hepatic tumors were stained overnight (at 4°C) for CD31/PECAM-1 (PharMingen) after acetone fixation as described above. Slides were rinsed with PBS (three times for 3 min each time) and incubated for 10 min in protein-blocking solution (5% goat serum) and washed with PBS before the addition of Texas Red-conjugated rat anti–CD31 secondary antibody (1:200; Jackson ImmunoResearch Laboratories) and subsequent incubation for 1 h at room temperature under light protection. Antibodies were washed off with PBS (three washes for 3 min each), and slides were blocked with nonspecific goat antimouse IgG Fab fragment (Jackson ImmunoResearch Laboratories), diluted 1:10 in protein block, for 1 h at room temperature to reduce background staining for the subsequent double staining procedure. After another rinsing cycle with PBS (three times for 3 min each time), slides were incubated for 10 min in protein-blocking solution. For pericyte staining (pericytes were defined as α-SMA-positive cells in direct contact with ECs), tumor sections were incubated overnight (at 4°C) with mouse anti-α-SMA.
(DAKO; 1:2000 in protein block solution). The antibody was then rinsed off with PBS, and protein-blocking solution was applied for 10 min. Alexa 488 (green; Jackson ImmunoResearch Laboratories) rabbit antimouse secondary antibody (1:200 in protein block solution) was added for 1 h at room temperature. Slides were rinsed in PBS, and nuclei were stained with Hoechst dye (1:2000) for 2 min. Slides were analyzed with an epifluorescence microscope equipped with narrow bandpass excitation filters (Chroma Technology Corp., Brattleboro, VT) to individually select for green, red, and blue fluorescence. Images were captured with a CS810 Hamamatsu camera (Hamamatsu Photonics K.K., Bridgewater, NJ) mounted on a Zeiss Axiosplan microscope (Carl Zeiss Inc., Oberkochen, Germany) using Optimas image analysis software (Media Cybernetics, Silver Spring, MD). Images were further processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA). Double-stained slides were analyzed at ×200 magnification for the degree of pericyte/CD31 colocalization as described elsewhere (33). The degree of pericyte coverage was evaluated in 4 fields/tumor (2 mm inside the tumor-normal tissue interface) and rated as absent or full coverage (defined as covering >90% of the vessel). The average percentage of covered vessels relative to uncovered vessels was then calculated for each tumor.

To confirm that Ang-1 was overexpressed in hepatic tumors, immunohistochemistry detection of Ang-1 was analyzed using goat anti-Ang-1 antibody N-18 (Santa Cruz Biotechnology, Santa Cruz, CA). Frozen tumor sections were fixed and blocked with protein block solution as described above. The primary antibody was added at 1:100 dilution and incubated overnight at 4°C. Slides were processed and analyzed as described, except that Alexa 488 anti-goat antibody (1:500) was added as secondary antibody for 1 h.

**Immunofluorescent Analyses of Vessel Density in Gelfoam Plugs.** Frozen sections of the agarose-Gelfoam plugs were prepared and stained for CD31/PECAM-1 (PharMingen) as described above. For immunofluorescent analysis, sections were incubated with Texas Red-conjugated goat antirabbit secondary antibody (1:200) as described above. Vessels were counted under an epifluorescence microscope at four different hot spots in each Gelfoam plug at ×50 magnification as described in the previous paragraph.

**In Vivo Miles Permeability Assay.** To investigate the effects of Ang-1 overexpression by tumor cells on vascular permeability, an intradermal Miles assay was performed. CM from Ang-1-transfected or pcDNA-transfected HT29 cells was collected after a 48-h incubation in 1% FBS-modified Eagle’s medium at 80% cell density, centrifuged for 5 min at 350 × g, and filtered through a 0.22-μm filter (Corning Inc., Corning, NY). Nude mice (n = 4) received i.v. injection with sterile 0.5% Evans blue dye (200 μl) via the tail vein. Ten min later, mice were given intradermal injections into the dorsal skin at three different sites, one for CM-Ang-1, one for CM-pcDNA, and one for VEGF (10 ng/ml; R&D Systems Inc., Minneapolis, MN). The VEGF served as positive control for increased vascular permeability. The intradermal injections (50 μl) were made with a 30-gauge needle. Mice were killed 20 min after the intradermal injections by cervical dislocation after anesthesia had been induced with Nembutal. The dorsal skin of each mouse was harvested to permit visualization of intradermal dye leakage. To determine the relative degree of vascular permeability, two dimensions (a and b) of the elliptically appearing area of dye leakage were obtained at each injection site by an observer blinded to the experimental group, and the area was calculated with the formula a × b × π.

**Densitometric Quantification of Vascular Permeability.** Densitometric analysis was performed using the NIH Image Analysis software (V1.62) from the NIH (Bethesda, MD) as another means of quantifying the extent of dye leakage at the intradermal injection sites in each mouse. Digitally obtained images of the underside of the dorsal skin, including all injection sites, were converted to a gray-scale image, and dye density was analyzed at each site (the threshold was set individually for each dorsal skin flap but was constant for each mouse).

**EC Coculture and Tie-2 Phosphorylation Assay.** To verify functional overexpression of Ang-1 in transfected HT29 cells, Ang-1- and pcDNA-transfected HT29 cells were cocultured with HUVECs for 48 h using transwell culture dishes (Corning Inc.). ECs were then harvested in PBS, and protein was isolated for Tie-2 immunoprecipitation as described previously (17). Briefly, 600 μg of protein for each experiment were used for immunoprecipitation using rabbit Tie-2 antibody (sc-324) and A/G plus agarose (both from Santa Cruz Biotechnology). Protein was then separated on a denaturing 7.5% SDS-polyacrylamide gel for Western blot analysis of Tie-2 phosphorylation.

**RESULTS**

**Effect of Ang-1 Overexpression on Hepatic Tumor Growth.** To evaluate the effects of Ang-1 overexpression on tumor growth of human colorectal cancer cells at the most common metastatic site (the liver), Ang-1-transfected or pcDNA-transfected (control) HT29 cells were injected directly into the liver parenchyma of mice to form single hepatic tumors. After 37 days of tumor growth, livers were excised, and liver weight and tumor diameters were determined. A. overexpression of Ang-1 significantly reduced tumor burden (reflected by liver weight) relative to control (*, P < 0.05, Student’s t test). B. Volumes of Ang-1 tumors were significantly smaller (†, P < 0.05, Mann-Whitney U test) than those of tumors in the pcDNA group (tumor volume = width² × length × 0.5). Bars, SE.

Membranes were probed with mouse anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY), and Tie-2 phosphorylation levels were analyzed by densitometry. Membranes were additionally probed for Tie-2 to assure equal loading.

**Statistical Analyses.** All statistical analyses were done using InStat Statistical Software (V2.03; GraphPad Software, San Diego, CA), with P values of ≤0.05 considered to be statistically significant. Results of in vivo experiments were also tested for significant outliers using the Grubb’s test for assessing outliers (*) or the Mann-Whitney U test (for nonparametric data) as specified in the figure legends. Fisher’s test was applied for comparing the incidence of hepatic tumor formation.

**Effect of Ang-1 Overexpression on Hepatic Tumor Growth.** To evaluate the effects of Ang-1 overexpression on tumor growth of human colorectal cancer cells at the most common metastatic site (the liver), Ang-1-transfected or pcDNA-transfected (control) HT29 cells were injected directly into the liver parenchyma of mice to form single hepatic tumors. Mice with visible tumor spillage at the time of the injection were excluded from further analysis. The experiment was terminated after 37 days of tumor growth, when mice in the control group became moribund. All nine mice in the control group and 71% (five of seven) of the mice in the Ang-1 group developed liver tumors (P = 0.17). However, Ang-1 overexpression led to a marked reduction of hepatic tumor burden (liver weight; P < 0.05; Fig. 1A). Ang-1 overexpression in tumors also led to a significant decrease in tumor volume (P < 0.05; Fig. 1B). Representative photographs of excised livers are presented in Fig. 2. To confirm that Ang-1 secretion from
the experimental cell line had no direct effect on tumor cell proliferation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed as described previously (34). Growth rates of both Ang-1- and pcDNA-transfected cells were similar over 24 and 48 h.

Effect of Ang-1 Overexpression on Tumor Vessel Density and Tumor Cell Proliferation. Tumor sections were stained for CD31 (PECAM-1) to allow vessels to be counted. Microvessel density was significantly reduced in Ang-1-expressing tumors ($P < 0.03$) as compared with pcDNA tumors (Fig. 3A). To investigate whether Ang-1 overexpression has indirect effects on tumor cell proliferation (HT29 cells are Tie-2 negative by reverse transcription-PCR; data not shown), we evaluated the percentage of proliferating tumor cells by immunohistochemical staining for PCNA. Ang-1-overexpressing colon cancer tumors demonstrated significantly lower percentages of proliferating (PCNA-positive) tumor cells than did tumors in the pcDNA group ($P < 0.01$; Fig. 3B). Representative immunohistochemical images of H&E, CD31, and PCNA analysis are demonstrated for Ang-1 and pcDNA tumors in Fig. 4.

Expression of Ang-1 and Effect of Ang-1 on Pericyte Coverage of Tumor Vessels. Immunohistochemical analysis of Ang-1 expression in hepatic tumors confirmed Ang-1 expression in HT29-Ang-1 tumors and normal tissues (data not shown). In contrast, and consistent with previous studies (13), Ang-1 was not detected in mock-transfected cells. The effect of Ang-1 on tumor EC pericyte coverage was investigated by immunofluorescent double staining of tumor vessels (CD31; red) and pericytes (α-SMA; green cells) adjacent to ECs. Ang-1 overexpression in HT29 hepatic tumors significantly increased the degree of pericyte coverage in the Ang-1 group ($P < 0.01$) as compared with pcDNA tumors (Fig. 5A). In quantitative terms, 68% of tumor vessels in the Ang-1-transfected tumors were tightly associated with and surrounded by pericytes, but only 13% of tumor vessels in the pcDNA tumors showed the same degree of high coverage (Fig. 5B).

Paracrine Effect of Ang-1 Secretion on in Vivo Tumor Growth. To confirm the paracrine effect of Ang-1 on in vivo angiogenesis, Ang-1 and pcDNA cells were mixed at various ratios and injected into the liver as described above. After 35 days of tumor growth, mice in the 100% pcDNA group became moribund, and the experiment was terminated. Tumor cell injections in this study revealed large tumor masses in the control group, but the tumors derived from Ang-1-overexpressing cells led to several small tumors within the left lobe of the liver. This was likely due to diffusion through the sinusoidal spaces. In the control group, individual tumor masses coalesced to

Fig. 2. Effect of Ang-1 expression on hepatic colon cancer growth. Representative images of excised livers are shown for each group. All nine of the mice in the pcDNA group and five of the seven mice in the Ang-1 group developed liver tumors. Hepatic tumor volumes in the Ang-1 group were significantly smaller than those in the pcDNA control group ($P < 0.05$).

Fig. 3. Effect of Ang-1 overexpression on vessel density and tumor cell proliferation. Tumor sections were stained for CD31 for vessel counts and for PCNA for percentage of proliferating tumor cells. A, fewer vessels were present in Ang-1-transfected tumors than in pcDNA-transfected tumors ($*, P < 0.03$). B, fewer proliferating cells were present in the Ang-1-transfected group than in the control (pcDNA) group ($*, P < 0.01$, Student’s t test). Bars, SE.

Fig. 4. Immunohistochemical analysis of vessel density (CD31) and tumor cell proliferation (PCNA) in hepatic tumors. Tumor sections were stained with H&E, anti-CD31 antibody, and PCNA antibody as described in “Materials and Methods.” Images were obtained at $\times50$ (H&E, CD31) or at $\times100$ (PCNA) magnification.
form large tumors, whereas tumor growth was inhibited when Ang-1 cells were present. Therefore, when several tumors were present, the largest was used for evaluation. Tumor volumes from cell mixtures that contained either 50% or 10% Ang-1-transfected cells were significantly lower than tumor volumes in the 100% pcDNA group (P < 0.04 for both; Fig. 6). Similar to our first study, cell suspensions containing only Ang-1-transfected (100%) cells formed significantly smaller tumor volumes compared with controls (100% pcDNA; P < 0.04). Liver weights in the 100% Ang-1 group were again significantly lower compared with controls (100% pcDNA; P < 0.03; data not shown).

Effect of Recombinant Ang-1 on Nonneoplastic Angiogenesis.
To demonstrate that the inhibition of hepatic tumor growth was mediated by inhibition of angiogenesis, we studied the effects of recombinant human Ang-1 (Ang-1 TFD) on nonneoplastic angiogenesis in vivo. For this purpose, we used a Gelfoam in vivo angiogenesis assay in which agarose-Gelfoam sponges were soaked with either Ang-1 (1.0 μg Ang-1 TFD/μl PBS) or PBS alone (control) and implanted subdermally in mice. Significantly fewer microvessels were present in the Ang-1-soaked Gelfoam plugs than in the PBS control Gelfoam plugs (P < 0.02; Fig. 7, A and B).

Effect of Conditioned Media of Ang-1-transfected Cells on Vascular Permeability.
Finally, we investigated the effect of Ang-1 overexpression by human colon cancer cells on the permeability of resting vasculature by using an intradermal Miles in vivo permeability assay, using conditioned media of transfected cell lines. CM from Ang-1-transfected cells abrogated the pro-permeability effects mediated by tumor cell-derived growth factors. HT29 cells produce relatively high amounts of VEGF compared with other cell lines (35). ELISA of conditioned media from both control and experimental cells contained similar levels of VEGF protein. Conditioned media from Ang-1-transfected cells led to a significant decrease in the overall area of dye leakage (mean ± SE, 8.0 ± 0.9 mm²) as compared with
conditioned media from pcDNA-transfected cells (29.3 ± 3.7 mm²; P < 0.05). Densitometric analysis of digital images of all injection sites confirmed that Evans blue dye density at the CM-Ang-1 injection sites was significantly less than that at the CM-pcDNA injection sites (P < 0.01; Fig. 8).

Effect of EC Coculture on Tie-2 Phosphorylation. To verify that Ang-1-transfected HT29 cells produce functionally relevant amounts of Ang-1, ECs were cocultured for 48 h with either Ang-1- or pcDNA-transfected colon cancer cells. The effect on Tie-2 phosphorylation was assessed by immunoprecipitation and Western blot analysis. By densitometry, there was a ∼2-fold increase in Tie-2 phosphorylation in HUVECs exposed to Ang-1-transfected cells, compared with Tie-2 phosphorylation levels in HUVECs cocultured with pcDNA HT29 cells (data not shown).

DISCUSSION

We report here three in vivo effects of Ang-1 in angiogenesis and regulation of vascular permeability: (a) overexpression of Ang-1 by HT29 human colon cancer cells inhibited tumor angiogenesis and the growth of hepatically implanted tumor cells in mice; (b) Ang-1 inhibited nonneoplastic angiogenesis in an in vivo angiogenesis assay; and (c) Ang-1 abrogated the pro-permeability effects of tumor cell-derived growth factors. The antiangiogenic effect of Ang-1 may have been mediated by the recruitment of periendothelial supporting cells (pericytes), leading to an overall vessel stabilization.

The functional complexity of Angs in the regulation of angiogenesis and in their effects on tumor growth is mirrored by conflicting reports on the in vivo effects of Ang-1 and Tie-2 activation (18–20, 22–24, 27, 36). Initially, Holash et al. (26) elegantly demonstrated the importance of coordinated induction of Angs and VEGF in tumor angiogenesis. Several studies have suggested that Ang-1 may be, in general, proangiogenic (6, 10, 11, 18, 24). Increased neoangiogenesis by Ang-1 was demonstrated in transgenic mouse models, where Ang-1 overexpression by keratinocytes, in combination with endogenous VEGF expression, led to increased dermal vascularization in mice, suggesting that VEGF and Ang-1 play coordinated and complementary roles. Thus, it was suggested that Ang-1 be used in combination with VEGF for promoting therapeutic angiogenesis (11, 18). The importance of cooperation of Ang-1 and VEGF for promotion of angiogenesis has been demonstrated in several malignant and nonmalignant models of angiogenesis (6, 10). However, until recently, only a few reports were available on the role of the Angs in tumor angiogenesis. In contrast to our previous report (27) on the effects of imbalances in Ang-1 and -2 expression in colon cancer cells, where Ang-1 overexpression inhibited angiogenesis and growth of xenografted tumors, Shim et al. (24) demonstrated that antisense Ang-1 mRNA expression by HeLa cervical adenocarcinoma cells inhibited angiogenesis and growth of xenografted tumors in immunodeficient mice. A different approach to Ang-1 inhibition was used by Lin et al. (19), who demonstrated that a soluble Tie-2 receptor could decrease angiogenesis and tumor growth of murine melanoma and mammary tumors when delivered by an adenoviral vector.

Recently, several studies suggested that overexpression or administration of Ang-1 may inhibit both neoplastic and nonneoplastic angiogenesis (17, 25, 36–37). Joussef et al. (37) investigated the effects of intravitreal Ang-1 application on retinal vascularization in diabetic rats. In that study, Ang-1 decreased retinal neovascularization and normalized VEGF levels. Similar results were found when Ang-1 was delivered systemically by an adenoviral vector (37). A blunted proangiogenic effect of VEGF by Ang-1 was also described by Visconti et al. (38) in a transgenic mouse model of cardiac-specific expression or coexpression of Ang-1, Ang-2, or VEGF. In our previous studies, we were able to demonstrate that imbalances in Ang expression may regulate growth and angiogenesis of human colon cancer. Ang-1 overexpression significantly inhibited tumor angiogenesis in that xenograft model (27). In the present study, our results again showed that overexpression of Ang-1 significantly reduced tumor growth (79%) and neovascularization (25%), this time in a model of colorectal cancer growing at the preferred site for metastases (i.e., liver). Additionally, by using various mixtures of cell suspensions of Ang-1- and pcDNA-transfected cells, we were able to demonstrate for the first time that Ang-1 secreted by tumor cells may impact the growth of tumor cells that do not express Ang-1. In this experiment, a mixture of 10% Ang-1-transfected cells with 90% pcDNA-transfected cells was sufficient to significantly reduce tumor growth in this group compared with the 100% pcDNA group. This suggests that adding Ang-1 to the tumor microenvironment may significantly inhibit the angiogenic process, resulting in an overall inhibition of tumor growth. The antiangiogenic effect of Ang-1 observed in our study may be mediated in part by increased periendothelial support by pericytes (high pericyte coverage in Ang-1 tumors), resulting in overall vessel stabilization and thereby inhibition of initiation of tumor angiogenesis. Because HT29 cells do not express Tie-2, the observed effects of Ang-1 expression on tumor growth result from effects on ECs and periendothelial cells rather than from effects on tumor cells themselves. However, Carlson et al. (39) recently demonstrated that Ang-1 may also interact with the extracellular matrix and mediate certain effects through a Tie-2-independent mechanism that involves various integrins. It rather unlikely that this mechanism plays a significant role in our tumor model because most integrins are absent on HT29 cells. In apparent contrast to our findings are those from Stratman et al. (40), who investigated the effects of Tie-2 inhibition in breast cancer cell lines that express various levels of Tie-2. Their results showed that transfection of a dominant-negative Tie-2 construct led to 15% growth inhibition in a Tie-2-negative cell line and 57% growth inhibition in a Tie-2 positive cell line, respectively. However, these observed effects are difficult to explain.

Fig. 8. Effect of conditioned media of Ang-1-transfected or pcDNA-transfected cells on vascular permeability. CM from transfected cells was collected in serum-reduced MEM after 48 h of culture, centrifuged, and filter-sterilized. The effects of this CM on vascular permeability were investigated with an intradermal Miles assay using Evans blue dye (0.5%); VEGF (10 ng/ml) served as positive control. Densitometry of harvested dorsal skin (20 min after intradermal injection) showed significantly lower dye density at CM-Ang-1 injection sites than at CM-pcDNA or VEGF injection sites (*, P < 0.01, Student’s t test). Representative images of the skin from one mouse are shown. Bars, SE.
Ang-1 also inhibited nonneoplastic neovascularization, as demonstrated by our in vitro angiogenesis assay with a novel recombinant Ang-1 (Ang-1 TFD; Ref. 31). The finding that the Gelfoam plugs were negative for α-SMA expression (data not shown) suggests that Ang-1 has a direct inhibitory effect on ECs in vivo.

Our findings are supported by the results of a recent study in which Ang-1 overexpression by MCF-7 breast cancer cells resulted in stabilization of blood vessels associated with the tumor edge (25). In that study, tumor cell proliferation decreased significantly in the presence of Ang-1 and prevented vessel dilatation and dissociation of smooth muscle cells from existing vessels, which resulted in reductions in xenografted tumor growth. On the basis of results from their Matrigel in vivo assay, in which Ang-1 increased mesenchymal cell infiltration, Tian et al. (25) concluded that vascular stabilization by Ang-1 accounts for the inhibition of tumor growth. They also demonstrated that Tie-2 was expressed on smooth muscle cells in culture (25). In a previous study, Haynes et al. (36) also demonstrated that Ang-1 overexpression in MCF-7 human breast cancer cells caused a significant retardation in tumor growth despite the high coexpression of a potent angiogenic growth factor (fibroblast growth factor-1). The same growth-inhibitory effect (70% reduction) by Ang-1 was observed by Hawighorst et al. (17) in stable transfected human squamous cell carcinoma cells. Those authors did not detect changes in vessel density but confirmed a significant increase in pericyte-covered vessels in Ang-1-transfected tumors (17). Results from our study expand these findings, showing that Ang-1 in the tumor microenvironment may also recruit pericytes into hepatic metastasis. Sundberg et al. (41) recently described that pericytes express Ang-1 at later stages of the angiogenic process, leading to further vessel stabilization and maturation of the tumor neovascular network. Taken together, these studies suggest that continuous Tie-2 activation on ECs may lead to increased vessel stabilization, thereby making the vasculature less susceptible to proangiogenic factors such as VEGF.

Vessel stabilization by Ang-1 is associated with decreased vascular permeability. In our in vivo permeability assay, Ang-1 levels in CM from Ang-1-transfected cells abrogated the increase of plasma leakage (dye leakage) caused by tumor cell-derived growth factors. Similar results were obtained with CM from transfected KM12L4 cells [high constitutive VEGF expression (35)], suggesting that Ang-1 is an important mediator of vascular stabilization and permeability and may override VEGF-mediated vessel leakage (42). This phenomenon has been described by other groups who have investigated the effects of Ang-1 on vascular permeability and vessel stabilization (11, 12). Thurston et al. described anti-permeability properties of Ang-1 in two different studies (11, 12), one evaluating the effect of VEGF on plasma leakage of adult vasculature, and another with a transgenic mouse model in which both Ang-1 and VEGF were overexpressed. In the mouse study, coexpression of Ang-1 and VEGF resulted in the formation of leakage-resistant vessels (11). The authors also showed that acute administration of Ang-1 protected adult vasculature from leakage mediated by VEGF and inflammatory cytokines (12). The molecular mechanism of this regulatory effect was recently described by Gamble et al. (43), who showed that administration of recombinant Ang-1 supported the localization of a cell adhesion molecule (PECAM-1) into junctions between ECs, thereby strengthening these junctions.

In conclusion, our study indicates that Ang-1 expression or administration may negatively regulate angiogenesis and decrease vascular permeability by stabilizing ECs and increasing periendothelial support. Thus, Ang-1 is an important mediator of neoplastic and nonneoplastic angiogenesis; however, its precise role in this process remains to be elucidated. Sequential expression of Ang-1, Ang-2, and VEGF has been shown to be crucial for successful angiogenesis (9, 10). Therefore, any interruption or disturbance in this balanced expression will probably affect the angiogenic process significantly. Such a disturbance could occur at the level of continuous Tie-2 activation (by Ang-1) or by Tie-2 interruption (soluble Tie-2, Tie-2 receptor antagonists). Results of future studies will be required to provide further insight into this complex process. Thus far, our findings suggest that Ang-1 might be useful as an antiangiogenic or anti-permeability agent in the treatment of metastatic colorectal cancer.

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Angiopoietin-1 expression reduces tumor angiogenesis


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Angiopoietin-1 Inhibits Vascular Permeability, Angiogenesis, and Growth of Hepatic Colon Cancer Tumors

Oliver Stoeltzing, Syed A. Ahmad, Wenbiao Liu, et al.


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