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Activation of the Tie2 Receptor by Angiopoietin-1 Enhances Tumor Vessel Maturation and Impairs Squamous Cell Carcinoma Growth

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The distinct roles of angiopoietin (Ang)-1 and Ang2, counteracting ligands for the endothelium-specific Tie2 receptor, in tumor development and progression have remained poorly understood. We investigated the expression of Ang1 and Ang2 during multistep mouse skin carcinogenesis and in human squamous cell carcinoma (SCC) xenografts. Expression of Ang2, but not of Ang1, was up-regulated in angiogenic tumor vessels already in early stages of skin carcinogenesis and was also strongly increased in SCCs. Stable overexpression of Ang1 in human A431 SCCs resulted in a more than 70% inhibition of tumor growth, associated with enhanced Tie2 phosphorylation levels, as compared with low levels in control transfected tumors. No major changes in the vascular density, vascular endothelial growth factor mRNA and protein expression, and vascular endothelial growth factor receptor-2 phosphorylation levels were observed in Ang1-expressing tumors. However, the fraction of tumor blood vessels with coverage by α-smooth muscle actin-positive periendothelial cells was significantly increased, indicative of an increased vascular maturation status. These findings identify an inhibitory role of Ang1/Tie2 receptor-mediated vessel maturation in SCC growth and suggest that up-regulation of its antagonist, Ang2, during early-stage epithelial tumorigenesis contributes to the angiogenic switch by counteracting specific vessel-stabilizing effects of Ang1. ***(Am J Pathol 2002, 160:1381–1392)***

Growth and metastasis of malignant tumors depend on the formation of new blood vessels, providing oxygenation and nutrient perfusion, as well as removal of waste products. To stimulate angiogenesis, tumor cells secrete proangiogenic growth factors that overcome other forces that keep existing vessels quiescent and stable.[1] Increasing evidence indicates that vascular endothelial growth factor (VEGF) and its endothelial tyrosine kinase receptors are key regulators of tumor angiogenesis,[2–4] and several reports demonstrated that inhibition of VEGF signaling resulted in reduced tumor growth and tumor neovascularization.[4–6]

Recently, a novel endothelial-specific receptor tyrosine kinase, Tie2, was identified that has been implicated in vascular maintenance as well as in angiogenesis.[7–9] Angiopoietin-1 (Ang1) specifically induces tyrosine phosphorylation of Tie2, whereas Ang2, a second ligand for the Tie2 receptor, has been identified as a naturally occurring antagonist for Ang1, blocking its ability to induce Tie2 tyrosine kinase activity in endothelial cells.[10] The angiopoietin/Tie2 system acts in a complementary manner with VEGF and plays an important role in late stages of vascular development.[11] Thus, in mouse embryos lacking either Ang1 or Tie2, the early stages of VEGF-dependent vascular development occur rather normally and result in the formation of a primitive vasculature, whereas maturation and stabilization of the primitive vasculature are severely perturbed.[12–14] Consistent with its proposed role as an Ang1 antagonist, transgenic overexpression of Ang2 in endothelial cells results in lethal embryonic defects comparable to those observed in Ang1- and Tie2-deficient mice.[10] Increasing evidence suggests that the mechanism of action of angiopoietins involves interactions between endothelial cells and pericytes.[15] Ang1 engagement of Tie2 has been proposed to stabilize mature blood vessels in the adult vasculature by promoting the interaction between endothelial cells and supporting periendothelial cells. In contrast, Ang2 has

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been thought to block the constitutive stabilizing effects of Ang1, thereby facilitating the angiogenic response to VEGF, or vessel regression in the absence of VEGF.[10,15]

There is increasing evidence that the angiopoietin/Tie2 system also plays an important role in tumor angiogenesis; however, the distinct biological effects of Ang1 and Ang2 on tumor growth and angiogenesis have remained controversial[16–20] and the regulation of expression of both factors during the consecutive stages of multistep carcinogenesis has remained unknown. In this study, we present evidence for an inhibitory role of Ang1-mediated Tie2 activation on squamous cell carcinoma (SCC) growth. Expression of Ang2, but not of Ang1, was strongly up-regulated in angiogenic tumor vessels during the consecutive stages of mouse skin carcinogenesis. Overexpression of Ang1 in A431 SCCs enhanced Tie2 receptor phosphorylation *in vivo* and resulted in a significant inhibition of tumor growth whereas overexpression of Ang2 did not modulate Tie2 phosphorylation and accordingly failed to affect tumor growth and angiogenesis. Moreover, Tie2 engagement by Ang1 significantly increased the fraction of mature blood vessels in A431 tumors, suggesting an important role of the maturation status of the tumor vasculature in tumor progression.

Materials and Methods

Chemical Skin Carcinogenesis Protocol

For tumor initiation, 25 μg of 7,12-dimethylbenz(d)anthracene (DMBA) (Sigma, St. Louis, MO) dissolved in 200 μl of acetone, were topically applied to the shaved back skin of 8-week-old female FVB mice, followed by weekly topical application of 5 μg of the tumor promoter PMA (Sigma) throughout 20 weeks as described.[21] Skin and tumor samples were embedded in OCT compound and frozen in liquid nitrogen for cryostat sectioning.

In Situ *Hybridization*

In situ hybridization was performed on 6-μm-thick cryostat sections with sense and anti-sense single-stranded ^{35}S-labeled RNA probes for Ang1 and Ang2 as described.[22]

Cell Culture

The human SCC cell line A431 was obtained from the American Type Culture Collection (Rockville, MD) and was maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine serum, 4.5 mg/ml glucose, 2 mmol/L L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Inc.). Human dermal microvascular endothelial cells (HDMECs) were isolated from neonatal human foreskins and were cultivated as described.[23]

Cell Transfection

A 2.0-kb full-length human Ang1 cDNA[24] and a 2.3-kb full-length human Ang2 cDNA,[10] were kindly provided by Dr. George D. Yancopoulos (Regeneron Pharmaceuticals Inc., Tarrytown, NY). Vectors for stable expression of human Ang1 and Ang2 were generated by subcloning of a 2.0-kb Ang1 cDNA and of a 2.3-kb Ang2 cDNA, comprising the complete coding sequences, into the *Xho*I (Ang1) or *Eco*RI (Ang2) sites of the pCDNA3.1Zeo(−) expression vector (Invitrogen, San Diego, CA) that contains a CMV-enhancer-promoter and a Zeocin selection cassette. The sequences and correct orientation were verified by restriction mapping and by direct sequencing using the Sanger dideoxy method. A431 cells were stably transfected with pCDNA/Ang1, pCDNA/Ang2, or pCDNA vector control, respectively, by using the SuperFect transfection reagent (Qiagen, Chatsworth, CA) and 10 μg of each plasmid according to the manufacturer's recommendations. Stably transfected clones were selected in medium containing 250 μg/ml of Zeocin (Invitrogen) for 3 to 4 weeks, and 35 clones for each construct were isolated and analyzed for Ang1 and Ang2 expression.

Cell Growth and Apoptosis Assays

To analyze anchorage-dependent cell growth, 5×10^4 A431 cells were plated in duplicate into 100-mm culture dishes, and the total cell numbers per dish were determined after 1 day (N_0) and after 7 days (N) at the end of the growth period (t) by using a hemocytometer. The cell-doubling time (T) was calculated by using the following formula: $T = \ln 2 \times t/\ln N/N_0$. Anchorage-independent cell growth was measured using a soft agar assay as described.[25] Briefly, 1×10^4 control-transfected or parental A431 cells, or cells transfected with Ang1 or Ang2 were transferred into six 35-mm cell-culture dishes with 2-mm grids (Nunc, Naperville, IL), and colonies were counted after 8 days. Apoptosis induced by serum withdrawal was studied in subconfluent A431 cell clones after 6 days of culture in serum-free medium. The percentage of apoptotic cells was determined as described,[26] using the Fluorescein-FragEL DNA fragmentation kit (Oncogene, Cambridge, MA) according to the manufacturer's instructions, and a Becton-Dickinson (Franklin Lanes, NJ) FACS-Scan.

Northern Blot Analysis

Total cellular RNA was isolated from confluent cell cultures and tumor tissues using the RNeasy kit (Qiagen). Ten μg of RNA were fractionated by electrophoresis on 1% agarose formaldehyde gels and were transferred to Biotrans nylon-supported membranes (ICN Pharmaceuticals, Costa Mesa, CA) as described.[27] ^{32}P-radiolabeled cDNA probes were prepared with a random primed synthesis kit (Multiprime; Amersham, Arlington Heights, IL).

mRNAs for Ang1, Ang2, and VEGF were detected with a 2.0-kbp human Ang1 cDNA, a 2.3-kbp human Ang2 cDNA, or a 204-bp human VEGF cDNA that recognizes all known VEGF variants.[28] A human 36B4 cDNA probe[29] was used as a control for equal RNA loading. Blots were washed at high stringency and exposed to X-OMAT MR film (Kodak, Rochester, NY) for varying times.

Receptor Phosphorylation Assays

To analyze the biological activity of transfected Ang1 and Ang2, an *in vitro* Tie2 receptor phosphorylation assay was used. Conditioned media (CM) were obtained from confluent stably transfected A431 cells grown for 48 hours in serum-free medium and were concentrated 50-fold by ultrafiltration (10,000 MWCO; Amicon, Inc., Beverly, MA). Protein concentrations were determined using the Bio-Rad protein assay (BioRad, Hercules, CA). HDMECs grown to 80% confluence and serum-starved overnight were left unchallenged or were challenged for 10 minutes with recombinant Ang1* (150 ng/ml), recombinant Ang1*[10] (150 ng/ml) plus recombinant Ang2 (rAng-2, 500 ng/ml), with CM obtained from Ang1 or vector control transfectants, or with recombinant Ang1* plus CM harvested from Ang2 transfectants, respectively. Recombinant Ang1* and recombinant Ang2[10] were generously provided by Dr. George D. Yancopoulos. Cells were solubilized in lysis buffer (1% Triton X-100, 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, and 10 μg/ml of aprotinin), and Tie2 was immunoprecipitated from the soluble fraction using a polyclonal antibody reactive with the carboxyl terminus of Tie2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C overnight. After incubation with protein A-Sepharose (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), immunocomplexes were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Bio-Rad). Tie2 phosphorylation was analyzed by probing the membranes with a monoclonal anti-phosphotyrosine antibody (PY-20; ICN Biomedicals, Inc., Aurora, OH). The same membranes were then stripped using TBST (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween 20, pH 2.4) overnight at room temperature and were reprobed for Tie2 with a rabbit anti-Tie2 antibody (Santa Cruz Biotechnology, Inc.). *In vivo* receptor phosphorylation assays were performed as recently described.[30] Briefly, samples obtained from three different vector control-transfected and Ang1- or Ang2-transfected A431 tumors were pooled and homogenized in RIPA lysis buffer containing protease inhibitors as above. Protein samples were immunoprecipitated using antibodies against Tie2 (RG133; kindly provided by Dr. Peter Maisonpierre, Regeneron Pharmaceuticals, Inc.) or mouse VEGFR-2 (flk-1; Santa Cruz Biotechnology, Inc.). Immunocomplexes were recovered on protein G-Sepharose and separated by 7.5% SDS-PAGE, transferred onto blotting membrane, and then probed with antibodies against phosphotyrosine (4G10; Upstate Biotechnology, Inc., Lake Placid, NY), Tie2 (Santa Cruz Biotechnology, Inc.), or VEGFR-2 (Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were visualized using a chemiluminescence detection system (ECL; Amersham). Levels of Tie2 phosphorylation were quantified by densitometry and were normalized to the expression levels of Tie2.

Reverse Transcriptase-Linked Polymerase Chain Reaction (RT-PCR)

RT-PCR of Tie1 and Tie2 mRNA expression in A431 clones was performed using 0.2 μg of total RNA as a template and reagents supplied by the GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA). For PCR amplification, the following primer sequences were selected from different exons of the human Tie1 and Tie2 genes: Tie1, 5′-TTTAACCCTGGTGTGCATCC-3′ (sense) and 5′-CCGCAGAAAATCTAGCAGGT-3′ (anti-sense; 451-bp PCR product); Tie2, 5′-AGTTCGAGGAGAGGCAATCA-3′ (sense) and 5′-CCGAGGTGAAGAGGTTTCCT-3′ (anti-sense; 290-bp PCR product). The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Stratagene, La Jolla, CA) were 5′-CCACCCATGG CAAATTCCATGGCA-3′ (sense) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (anti-sense; 600-bp PCR product). HDMECs at passage 6 were used as a positive control. Cycling parameters were as follows: Tie1 and Tie2: denaturation, 94°C for 30 seconds; annealing, 53°C for 30 seconds; extension, 65°C for 30 seconds, with 35 cycles. GAPDH: denaturation, 94°C for 45 seconds; annealing 60°C for 45 seconds; extension, 72°C for 90 seconds, with 35 cycles. PCR products were electrophoresed in 3% agarose gels and were visualized by ethidium bromide staining. For analysis of Ang1 and Ang2 mRNA expression in tumors real-time RT-PCR was performed using 100 ng of RQ-DNase-treated total RNA per each reaction. The sequences of the primer/dual labeled probe sets were as follows: Ang1: 5′-TGTTAACAGGAGGATGGTGGTTT-3′ (sense),FAM-TGCTTGTGGCCCCTCCAATCTAAATG-TAMRA (dual-labeled probe), and 5′-GTTTTGTCCCGCAGTATAGAACATT-3′ (anti-sense). Ang2: 5′-GCCGCTCGAATACGATGACT-3′, FAM-TGCAGAGGCTGCAAGTGCTGGAGAA-TAMRA (dual-labeled probe), and 5′-CCACTGAGTGTTGTTTTCCATGA-3′ (anti-sense). Each reaction was performed in the presence of a GAPDH internal control detection system using the Taqman EZ RT-PCR Core Reagent (Applied Biosystems). All reactions were performed in triplicates using the ABI Sequence Detection System 7000 (Applied Biosystems).

Tumorigenicity Assay

Stably transfected A431 cells (2×10^6 in 100 μl of serum-free culture medium) were injected intradermally into both flanks of 8-week-old female Swiss (nu/nu) mice (five mice for each clone). Two vector-transfected clones, three Ang1- or Ang2-overexpressing clones, and the pa-

rental cell line were studied. The smallest and largest tumor diameter were measured weekly using a digital caliper, and tumor volumes were calculated using the following formula: volume = 4/3 × π × (1/2 × smaller diameter)^2 × 1/2 × larger diameter. Tumor data were analyzed using the two-sided unpaired *t*-test. Tumors were harvested after 3 weeks and were either embedded in OCT compound and frozen in liquid nitrogen for cryostat sectioning, or were fixed in formalin and embedded in paraffin for routine histology. For RNA extractions, tumors were snap-frozen in liquid nitrogen. In an additional experiment one vector-transfected clone, one Ang1-overexpressing clone, and one Ang2-overexpressing clone (2×10^6 cells/injection) or a 1:1 mixture of one vector control and one Ang1-transfected clone or a mixture of one Ang1-transfected and one Ang2-transfected clone (1×10^6 cells each clone/injection) were injected ($n = 10$ tumors per group). All animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Western Blot Analysis

Tumors were snap-frozen in liquid nitrogen and homogenized in lysis buffer (2% SDS, 50 mmol/L Tris, pH 7.4, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, and 10 μg/ml of aprotinin). Fifty μg of protein per sample were analyzed by denaturing SDS/PAGE and immunoblotted with a rabbit antibody against human VEGF (kindly provided by Dr. Donald R. Senger, Harvard Medical School, Boston, MA).

Computer-Assisted Morphometric Analysis of Tumor Vessels

Cryostat sections (6 μm) were stained with a rat anti-mouse platelet-endothelial cell adhesion molecule-1 (CD31) monoclonal antibody (Pharmingen, San Diego, CA) as previously described.[31] Representative sections obtained from five A431 tumors from each cell clone were analyzed using a Nikon E-600 microscope (Melville, NY). Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI), and morphometric analyses were performed using the IP LAB software (Scanalytics, Billerica, MA) as described.[32] Three different fields in each section were examined at ×10 magnification, and the number of vessels per mm^2, the vessel size, and the relative area occupied by tumor blood vessels within the tumors were determined. The two-sided unpaired *t*-test was used to analyze differences in microvessel density, vessel size, and total vascular area.

Indirect Immunofluorescence

Cryosections were fixed and stained as previously described,[4] using monoclonal antibodies against mouse CD31 (dilution, 1:50; Pharmingen), or against α-smooth muscle actin (1:100, α-SMA, Cl 14A; DAKO, Glostrup, Denmark), expressed by vascular smooth muscle cells and pericytes. The secondary antibodies, labeled with either Texas Red or fluorescein isothiocyanate (Jackson ImmunoResearch, West Grove, PA) were used at a 1:50 dilution. Sections were incubated, together with the secondary antibody, with 20 μg/ml of Hoechst bisbenzimide (Sigma Chemical Co.) for staining of cell nuclei. Specimens were mounted in Mowiol (Calbiochem, La Jolla, CA), and conventional fluorescent and light microscopy were performed on a Nikon E-600 microscope. Three independent fields per section obtained from each cell clone were analyzed, and the percentage of blood vessels associated with α-SMA-positive cells was defined as the vessel maturation index.[33] The two-sided unpaired *t*-test was used to analyze differences in the vessel maturation index.

Results

Up-Regulation of Ang2 Expression Is an Early Event during Multistep Carcinogenesis

We first investigated the regulation of Ang1 and Ang2 mRNA expression during chemically induced two-step mouse skin carcinogenesis by *in situ* hybridization. In normal skin, Ang1 was constitutively expressed at low levels by mesenchymal stroma cells in the vascularized dermis (Figure 1, A and D), whereas no Ang2 mRNA was detectable (Figure 1, G and J). The expression of Ang2 (Figure 1; G to L), but not of Ang1 (Figure 1; A to F), was strongly up-regulated throughout the consecutive stages of mouse skin carcinogenesis. Enhanced Ang2 expression signals were detected in endothelial cells of tumor-associated blood vessels in benign papillomas (Figure 1, H and K) and even more pronounced in SCC (Figure 1, I and L). Ang1 or Ang2 were not expressed by tumor cells in benign papillomas (Figure 1; B, E, H, and K) or in SCCs (Figure 1; C, F, I, and L). These results identified up-regulation of Ang2 mRNA expression as an early event during multistep carcinogenesis. We next investigated whether the switch to Ang2 expression also occurred in an established mouse model of SCC using intradermally transplanted human A431 SCC. A431 tumor cells did not express Ang1 (Figure 2, A and C) or Ang2 (Figure 2, B and D). Little Ang1 expression was detectable in mesenchymal stroma cells (Figure 2; A, C, and E), whereas Ang2 mRNA was strongly expressed by endothelial cells of tumor blood vessels (Figure 2; B, D, and F). Control hybridizations with sense riboprobes for both Ang1 (Figure 2, G and I) and Ang2 (Figure 2, H and J) revealed very low background signals. Taken together, these findings suggested that enhanced Ang2 expression might contribute to tumor progression and angiogenesis by blocking the constitutive stabilizing effects of Ang1.

Expression of Biologically Active Ang1 and Ang2 in A431 Cells

To investigate the distinct effects of Ang1 *versus* Ang2 on tumor growth and angiogenesis, A431 cells were stably

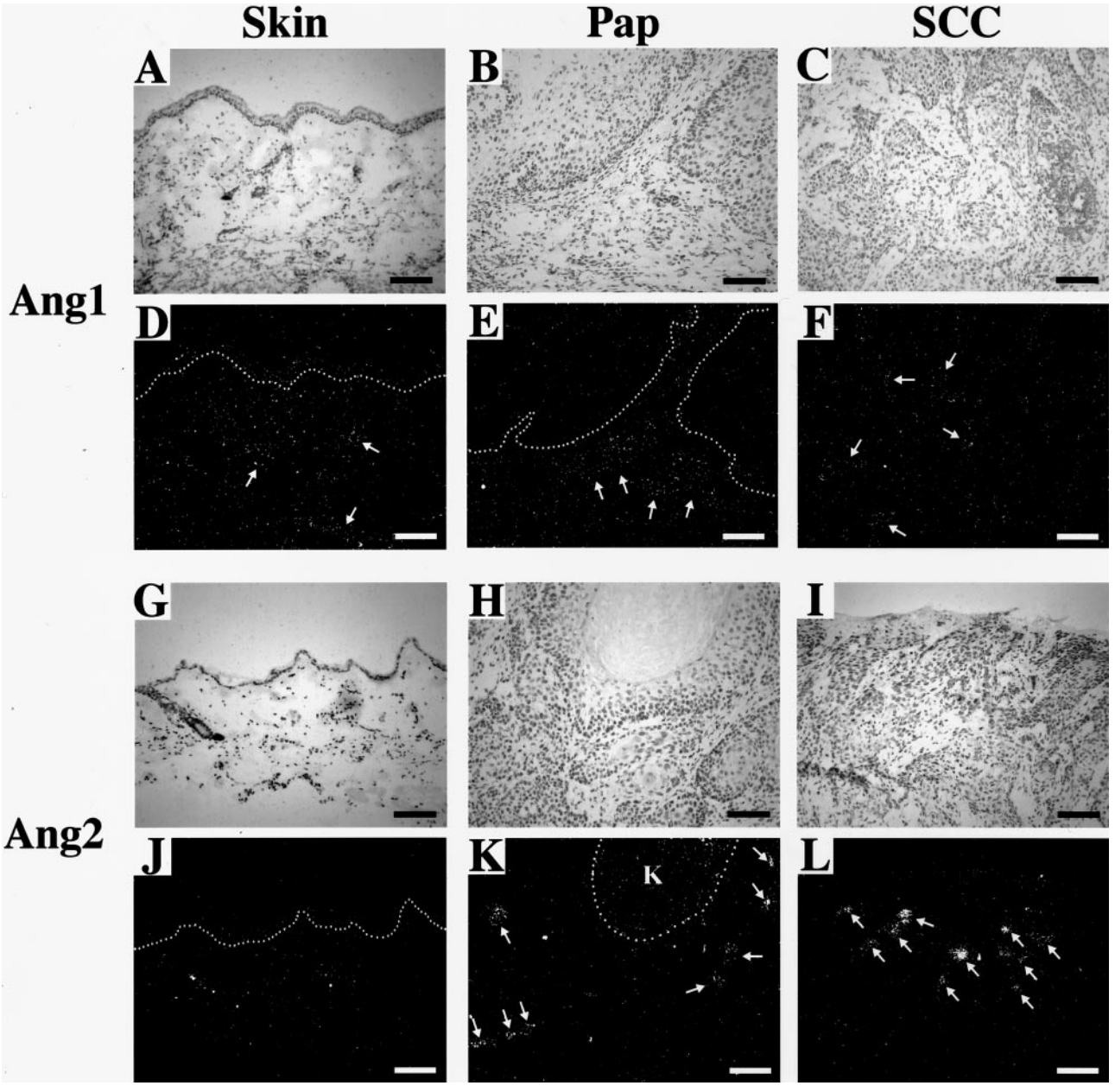

Figure 1. Up-regulation of Ang2 expression during skin carcinogenesis. *In situ* hybridization demonstrates low-level Ang1 mRNA expression in the dermis of normal skin (**A** and **D**) and in the mesenchymal stroma of a representative small papilloma (**B** and **E**) and SCC (**C** and **F**). In contrast, although Ang2 mRNA was barely detectable in normal skin (**G** and **J**), Ang2 mRNA was strongly expressed by tumor-associated endothelial cells (**arrows**) of benign papillomas (**H** and **K**) and even more pronounced in SCCs (**I** and **L**). Bright-field (**A–C** and **G–I**) and dark-field (**D–F** and **J–L**) micrographs. **E**, **F**, and **K:** The **dotted lines** delineate the border between epithelium and mesenchymal stroma. **K:** The **dotted line** delineates the border between keratin layer and tumor cells. Scale bars, 100 μm.

transfected to constitutively overexpress Ang1 or Ang2. As determined by Northern analysis, parental A431 cells and vector-transfected control clones did not express any detectable amounts of Ang1 or Ang2 mRNA *in vitro* (Figure 3A), whereas three different Ang1- and Ang2-transfected clones strongly expressed the mRNA for Ang1 or Ang2, respectively (Figure 3A). We next performed Tie2 receptor phosphorylation assays in HDMECs to analyze the biological activity of transfected Ang1 and Ang2. Recombinant Ang1* (150 ng/ml) induced tyrosine phosphorylation of Tie2. This induction was blocked by addition of recombinant Ang2 (500 ng/ml), confirming the functional activity of the assay (Figure 3B). CM obtained from Ang1-transfected A431 clones potently induced tyrosine phosphorylation of Tie2, as compared with CM obtained from A431 cells transfected with vector only (Figure 3B). In contrast, CM harvested from Ang2-overexpressing A431 clones potently blocked Ang1*-mediated Tie2 phosphorylation (Figure 3B). These findings confirmed the biological activity of Ang1 and Ang2 secreted by stable transfectants. They are in agreement with the proposed role of Ang1 acting as an agonist for the Tie2 receptor and of Ang2 acting as an Ang1 antagonist at the level of the Tie2 receptor.

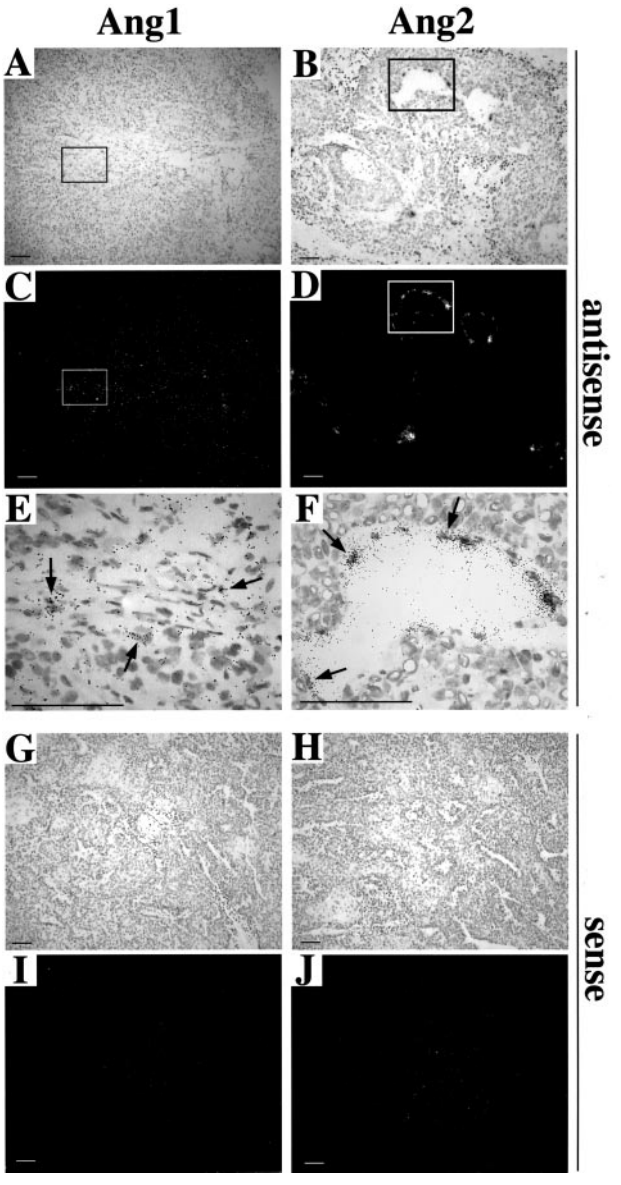

Figure 2. Enhanced Ang2 expression in A431 SCCs. In A431 SCCs, little Ang1 mRNA was detectable in the mesenchymal stroma (**A** and **C**), whereas Ang2 mRNA was strongly expressed by tumor blood vessels (**B** and **D**). **E:** High-power photomicrograph of **insets** in **A** and **C** showing low-level Ang1 expression associated with mesenchymal stromal cells (**arrows**). **F:** High-power photomicrograph of **insets** in **B** and **D** depicting strong Ang2 expression by endothelial cells (**arrows**). Hybridization with Ang1 sense (**G** and **I**) and Ang2 sense (**H** and **J**) control probes demonstrates low background signal. Bright-field (**A**, **B**, **E**, **F**, **G**, and **H**) and dark-field (**C**, **D**, **I**, and **J**) micrographs. Scale bars, 200 μm.

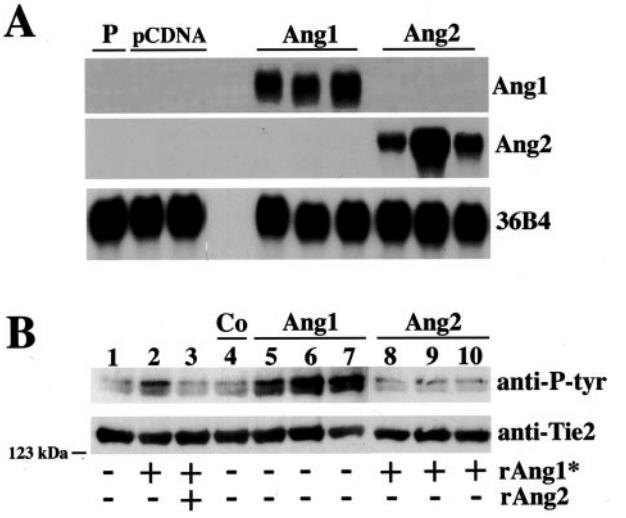

Figure 3. Expression of biologically active Ang1 and Ang2 in stably transfected A431 cells. **A:** Northern blot analysis confirmed Ang1 or Ang2 mRNA expression in three different stably transfected A431 clones, whereas no mRNA expression of Ang1 or Ang2 was detected in vector control clones (pCDNA) or in parental A431 cells (P). The blot was also probed with a 36B4 cDNA probe to control for loading. **B:** Tie2 receptor phosphorylation assays using human microvascular endothelial cells confirmed the biological activity of transfected Ang1 and Ang2. Recombinant Ang1* (**lane 2**) and CM obtained from three different Ang1 clones (**lanes 5–7**) induced Tie2 phosphorylation. CM obtained from a representative vector-transfected A431 clone (**lane 4**) did not induce Tie2 phosphorylation, as compared to unchallenged endothelial cells (**lane 1**). Recombinant Ang2 (**lane 3**) and CM harvested from three different Ang2 clones (**lanes 8–10**) blocked Tie2 phosphorylation induced by recombinant Ang1*. Tie2 was immunoprecipitated and analyzed by SDS-PAGE followed by Western blotting and detection with phosphotyrosine (anti-P-tyr) antibodies with subsequent stripping and reprobing of the membrane with antibodies for Tie2 (anti-Tie2).

Characterization of Tumor Cell Growth and Apoptosis in Vitro

Several *in vitro* assays were performed to detect potential phenotypic differences between control and Ang1- and Ang2-transfected A431 SCC clones. Anchorage-independent growth was determined to evaluate the maintenance of the transformed phenotype and was studied by determination of colony numbers in a soft agar assay. No significant differences in the number of colonies were observed between the different transfected A431 clones and parental A431 cells (Figure 4A). In accordance, cell-doubling times under anchorage-dependent culture conditions in Ang1- and Ang2-overexpressing clones were comparable to those observed in vector-transfected or parental A431 control cells (Figure 4B). RT-PCR analyses (Figure 4C) demonstrated the complete absence of Tie2 and orphan Tie1 receptor mRNA expression in vector-control and Ang1- and Ang2-transfected A431 cells, whereas expression of both Tie2 and Tie1 mRNA was clearly detected in cultured HDMECs used as positive control. To compare the susceptibility to induction of apoptosis, A431 clones were cultured under serum-free conditions for 6 days. No significant differences in the percentage of apoptotic cells were found between Ang1 (6.8% $\pm$ 1.0), Ang2 (8.7% $\pm$ 3.0), or control transfected clones (12.5% $\pm$ 6.3). Moreover, no alterations in cellular morphology were detected (data not shown).

Activation of Tie2 by Ang1 Suppresses Orthotopic SCC Growth

After intradermal injection into immunodeficient nude mice, parental A431 cells and vector-transfected control cells formed rapidly growing SCCs, reaching a volume of 1500 to 1800 mm^3 within 3 weeks (Figure 5A). In contrast, overexpression of Ang1 resulted in a significant inhibition of tumor growth by more than 70% ($P < 0.001$, Figure 5A). Even at 5 weeks after tumor injection, Ang1-overex-

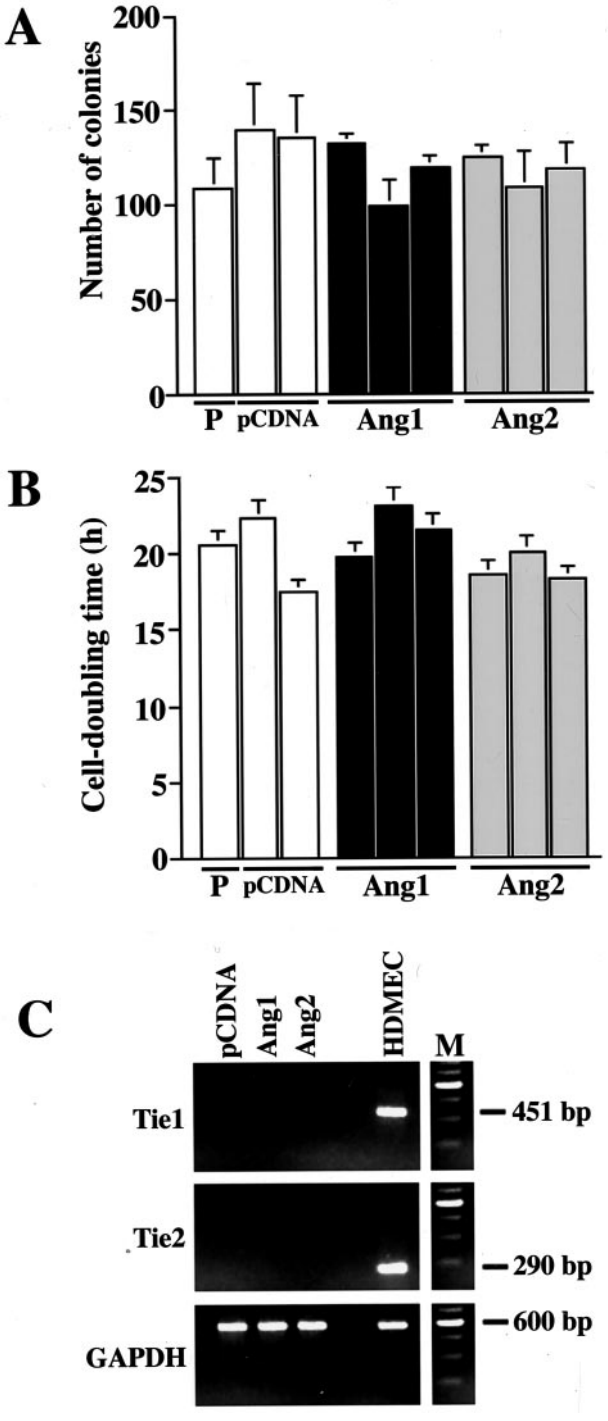

Figure 4. *In vitro* growth assays and RT-PCR analysis of Tie1 and Tie2 mRNA expression. **A:** No significant differences in anchorage-independent growth of three different Ang1- and Ang2-transfected A431 clones as compared with parental (P)- and vector (pCDNA)-transfected control cells. Mean values ± SD of two independent experiments. **B:** Cell-doubling times of Ang1- and Ang2-overexpressing clones under anchorage-dependent culture conditions in Ang1- and Ang2-overexpressing clones were comparable to those of control-transfected (pCDNA) or parental A431 cells (P). **C:** Ethidium bromide-stained gel of RT-PCR products demonstrating the complete absence of both Tie1 (**top**) and Tie2 (**middle**) mRNA expression in pCDNA vector control-transfected, and Ang1- and Ang2-transfected A431 cells. HDMECs expressed both Tie1 and Tie2 mRNA and served as positive control. Analysis for GAPDH mRNA expression was used as internal control (**bottom**). M, DNA marker.

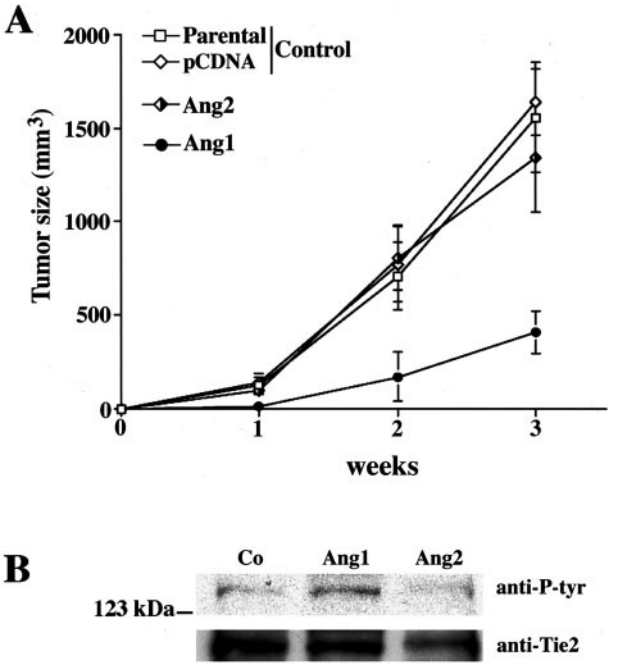

Figure 5. Ang1-mediated Tie2 activation inhibits *in vivo* tumor growth. **A:** Ang1 overexpression in A431 cells potently inhibited intradermal tumor growth as compared with parental A431 cells or vector-transfected control clones (pCDNA, $P < 0.001$). In contrast, overexpression of Ang2 did not result in any major difference of tumor growth, as compared with parental A431 cells or with vector-transfected control clones (pCDNA). Results are expressed as mean values ± SD of one parental, two control-transfected, three Ang1-transfected, and three Ang2-transfected cell clones for each time point. **B:** *In vivo* Tie2 phosphorylation assays demonstrate increased phosphorylation of the Tie2 receptor in Ang1-overexpressing tumors as compared with control tumors (Co). In Ang2-overexpressing tumors, Tie2 phosphorylation was comparable to that of control tumors. Tie2 was immunoprecipitated from tumor lysates and analyzed by SDS-PAGE followed by Western blotting and detection with phosphotyrosine (anti-P-tyr) or Tie2 (anti-Tie2) antibodies.

pressing tumors did not reach the size of control tumors at 3 weeks after injection (data not shown). Surprisingly, overexpression of Ang2 in A431 tumors had no effect on tumor growth (Figure 5A). Northern blot analysis of RNA extracted from representative tumors confirmed that Ang1- and Ang2-transfected tumors maintained their respective mRNA expression *in vivo* throughout the entire observation period (data not shown). By using quantitative real-time RT-PCR we found that the average expression of endogenous Ang2 in control tumors was 115-fold higher than the average expression of endogenous Ang1. However, in Ang1-transfected tumors, the average expression of recombinant Ang1 was more than 74-fold higher than the expression of endogenous Ang2. In Ang2-transfected tumors, the average expression of recombinant Ang2 was more than 9 times higher than the endogenous Ang2 expression. In accordance, *in vivo* Tie2 phosphorylation assays demonstrated that overexpression of Ang1 resulted in increase of phosphorylation levels of the Tie2 receptor *in vivo* as compared with vector-transfected controls (2.6-fold induction, Figure 5B). No major differences in the expression levels of Tie2 were found between control and Ang1-transfected tumors (Figure 5B). In Ang2-overexpressing tumors, the phosphorylation levels of Tie2 were comparable to those of

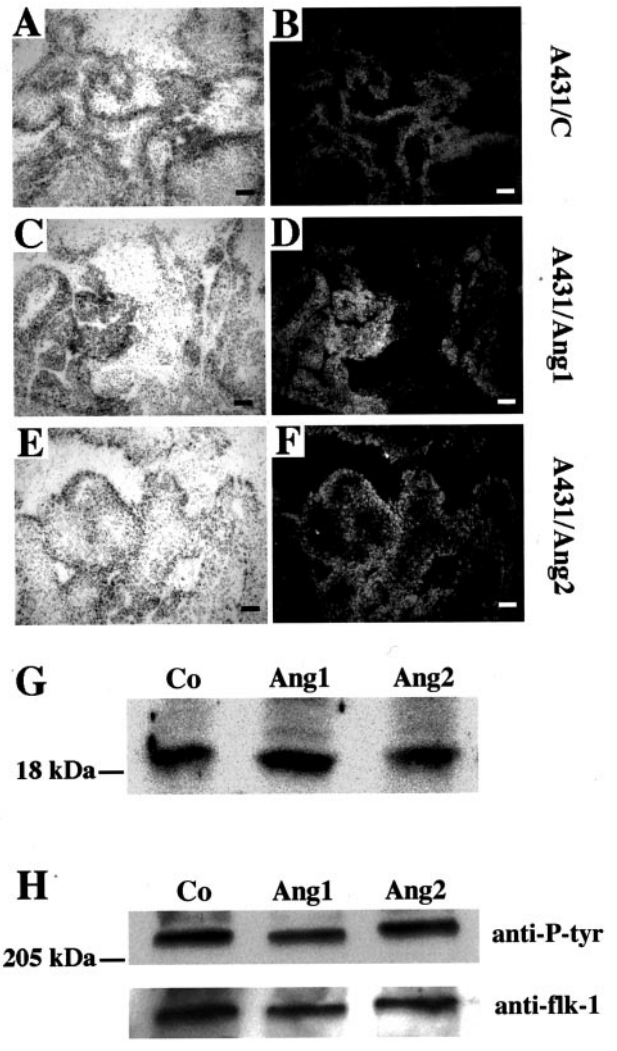

Figure 6. Comparable VEGF expression in control and Ang1- and Ang2-overexpressing tumors. *In situ* hybridization demonstrates comparable VEGF mRNA expression in representative control (**A** and **B**) and Ang1 (**C** and **D**)- or Ang2-overexpressing (**E** and **F**) A431 tumors. Bright-field (**A**, **C**, and **E**) and dark-field (**B**, **D**, and **F**) micrographs. **G:** No significant differences of VEGF protein levels were found in the different tumors as studied by Western blot analysis. **H:** *In vivo* VEGFR-2 (flk-1) phosphorylation assays revealed similar expression and phosphorylation levels of VEGFR-2 (flk-1) in control (Co) and Ang1- and Ang2-overexpressing tumors. VEGFR-2 was immunoprecipitated from tumor lysates and analyzed by SDS-PAGE followed by Western blotting and detection with phosphotyrosine (anti-P-tyr) or VEGFR-2 (anti-flk-1) antibodies. Scale bars, 100 μm.

control tumors (Figure 5B). These results demonstrated that Ang1-mediated activation of the Tie2 receptor resulted in inhibition of tumor growth.

Ang1 Overexpression Does Not Modulate VEGF Activity

VEGF and the angiopoietins play complementary and coordinated roles in angiogenesis.[11] We next studied whether the expression of VEGF was modulated by overexpression of Ang1 or Ang2. *In situ* hybridizations revealed that VEGF mRNA was expressed at comparable levels in the different A431 tumors (Figure 6; A to F). These findings were confirmed by Northern blot analysis (data not shown). Moreover, no significant differences of VEGF protein levels were found between control and Ang1- and Ang2-transfected tumors, as studied by Western blot analysis (Figure 6G). Importantly, immunoprecipitation studies of tumor lysates revealed similar expression and phosphorylation levels of VEGFR-2 (flk-1) in tumors of all types (Figure 6H).

Overexpression of Ang1 Results in Increased Maturation of the Tumor Vasculature

We analyzed the tumor-associated vasculature, visualized with an antibody to CD31. Immunostains showed that blood vessels were distributed rather uniformly throughout the viable tumor areas in all experimental groups (Figure 7A). Moreover, morphometric analyses of tumor-associated blood vessels did not reveal any significant differences of vascular density or average vessel size (Figure 7, B and C) between control tumors and Ang1- or Ang2-overexpressing tumors. Because the mechanism of action of angiopoietins may involve critical interactions between endothelial cells and pericytes,[11,15] we next investigated the effects of Ang1 and Ang2 overexpression on the maturation status of the tumor vasculature, using double-immunofluorescent stains for CD31 (tumor endothelial cells) and SMA (smooth muscle cells/pericytes). Ang1-overexpressing tumors exhibited an increased number of blood vessels surrounded by SMA-positive periendothelial cells, as compared with control and Ang2-overexpressing tumors (Figure 8; A to C). The maturation index, defined as the fraction of blood vessels associated with SMA-positive periendothelial cells, was significantly increased, by more than 50%, in Ang1-overexpressing tumors as compared with control tumors ($P < 0.05$; Figure 8D). No major differences were found between control and Ang2-overexpressing tumors (Figure 8D). These findings demonstrate that overexpression of Ang1 in tumors results in enhanced ratios of mature blood vessels, possibly by counteracting the destabilizing effects of endothelium-derived Ang2.

Ang2 Partially Rescues the Reduced Growth of Ang1-Overexpressing Tumors

To investigate whether Ang2 might rescue the reduced growth of Ang1-overexpressing tumors, we performed additional *in vivo* studies with mixtures of Ang1- and Ang2-overexpressing cells. In accordance with the results above, tumor growth was inhibited by overexpression of Ang1 whereas overexpression of Ang2 had no effect (Figure 9). The admixture of Ang2-overexpressing cells to Ang1-overexpressing cells (1:1) greatly diminished the inhibition of tumor growth mediated by Ang1 ($P < 0.001$). Admixture of vector control-transfected cells to Ang1 overexpressing only slightly diminished the inhibitory effects of Ang1.

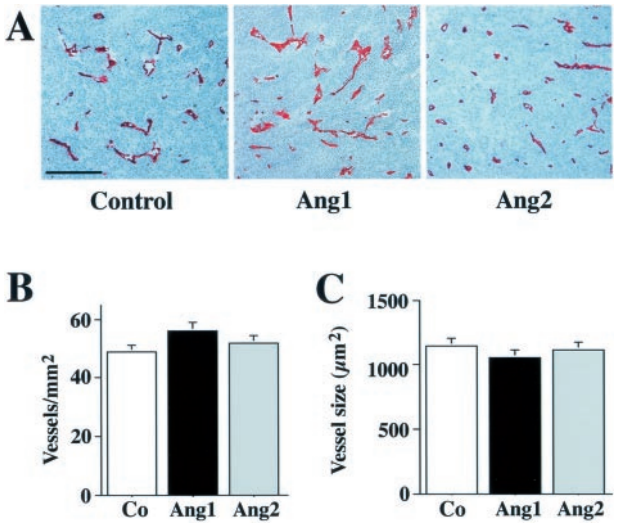

Figure 7. Morphometric analysis of tumor-associated blood vessels. **A:** CD31 immunohistochemistry demonstrates a rather uniform distribution of tumor-associated blood vessels throughout the viable tumor areas in control and Ang1- and Ang2-expressing tumors. Computer-assisted morphometric analyses of CD31-stained blood vessels did not reveal any significant differences in vascular densities (**B**) or average vessel sizes (**C**) in tumors derived from Ang1- and Ang2-overexpressing A431 cells as compared to control tumors (Co). CD31-stained blood vessels were evaluated in three different ×10 fields in sections obtained from five different tumors for each clone. Data are expressed as mean values ± SEM.

Discussion

Although increasing evidence indicates that the angiopoietin/Tie2 system plays an important role in tumor angiogenesis, the distinct biological effects of the Tie2 agonist Ang1 and its antagonist Ang2 have remained unclear. Here, we identify the switch to predominant Ang2 expression as an early event in multistep carcinogenesis. Consistent with previous data,[10] we observed constitutive expression of Ang1 in normal skin, indicating a potential role in blood vessel homeostasis. In contrast, little or no expression of Ang2 was detectable in normal skin. However, the expression of Ang2, but not of Ang1, was strongly up-regulated during carcinogenesis, indicating that the switch in the balance between Ang1 and Ang2 may play an important role in tumor angiogenesis and growth.[18,34] Combined with previous findings that identified induction of angiogenesis by VEGF as an early occurring, rate-limiting step in multistage skin carcinogenesis,[21,35,36] our results suggest that the normal dermal vascular network is maintained and stabilized by the constitutive expression of Ang1, whereas Ang2 collaborates with VEGF to induce tumor angiogenesis and to promote skin carcinogenesis.

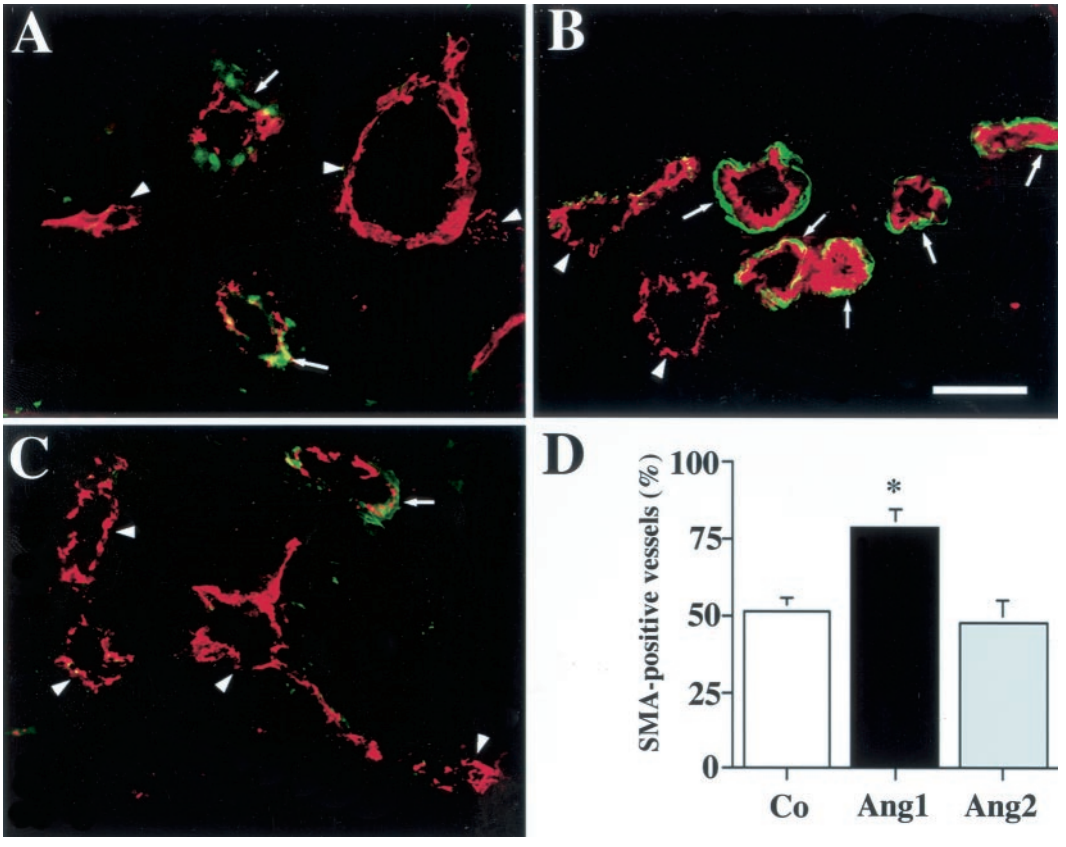

Figure 8. Ang1 overexpression in tumors results in increased vessel maturation. **A–C:** Double-immunofluorescence stains for CD31 (red) and α-SMA (green) distinguish vessels that are not associated with SMA-positive cells (red; **arrowheads**) from vessels associated with SMA-positive cells (**arrows**). Representative fields show increased coverage of tumor blood vessels by mural cells in Ang1-overexpressing tumors (**B**) as compared to control (**A**) or Ang2 (**C**)-overexpressing tumors. **D:** Significantly increased vessel maturation index (*, $P < 0.05$), defined as the percentage of vessels associated with SMA-positive periendothelial cells, in Ang1-overexpressing tumors, as compared with control tumors (Co). No significant differences were found between control tumors and Ang2-overexpressing tumors. Data are expressed as mean values ± SEM of three independent fields per section obtained from each cell clone. Scale bars, 50 μm.

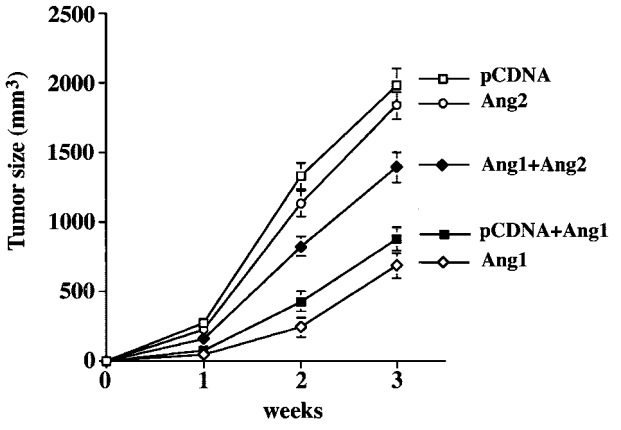

Figure 9. Ang2 partially rescues the inhibitory effect of Ang1 on tumor growth. Ang1 overexpression in A431 cells inhibited tumor growth as compared with vector-transfected control clones (pCDNA) whereas Ang2 overexpression had no effect. Admixture of Ang2-overexpressing cells to Ang1-overexpressing cells (1:1; Ang1+Ang2) greatly diminished the inhibition of tumor growth mediated by Ang1 ($P < 0.001$). Addition of vector control-transfected to Ang1-overexpressing cells (pCDNA+Ang1) only slightly diminished the inhibitory effects of Ang1. Results are expressed as mean values + SEM for each time point ($n = 10$ tumors per group).

Controversial findings have been reported regarding the effects of Ang1 *versus* Ang2 expression on the growth and angiogenesis of established tumors. Whereas Ang1 inhibited MCF-7 breast cancer growth,[16] recent studies in HT29 colon,[18] TA3 mammary, and Lewis lung carcinomas[17] did not detect major effects of Ang1 overexpression on *in vivo* tumor growth. In addition, overexpression of Ang2 has been found to either promote[18–20] or to inhibit[17] *in vivo* growth of various tumor xenografts. In these experimental tumor studies, the *in vivo* Tie2 phosphorylation levels in response to Ang1 and Ang2 overexpression had not been assessed.

To study the effects of *de novo* Ang1 or Ang2 expression on *in vivo* Tie2 phosphorylation, angiogenesis, and tumor growth, we made use of an established orthotopic xenograft mouse model,[25,32] using human A431 SCC cells. The A431 cell line was chosen because of the complete absence of Ang1 and Ang2 expression in these cells and because we found an expression pattern of Ang1 and Ang2 in A431 xenografts that was comparable to that observed during multistep mouse skin carcinogenesis. Our results demonstrate that overexpression of Ang1 promoted the interactions between endothelial cells and periendothelial cells in human SCCs. The observed increase in the percentage of mature blood vessels was consistent with the marked induction of Tie2 phosphorylation *in vivo*. These data are in agreement with the recently proposed vessel-stabilizing role of Ang1.[15] Whereas activated tumor endothelium may promote tumor progression,[4,37,38] our results indicate that increased maturation of tumor vessels may result in inhibition of tumor growth because overexpression of Ang1 potently inhibited the growth of A431 SCCs by more than 70%. The potential relevance of these findings to human disease is supported by the recently reported inverse correlation between the expression of Ang1 and the size of human thyroid tumors.[39]

The exact mechanisms by which Ang1-mediated vessel maturation impairs tumor growth remain to be established. Pericytes and perivascular smooth muscle cells have been shown to play major roles in maintaining vessel maturation and integrity.[12–14,40–42] Thus, as a result of the increased fraction of mature tumor vessels that are surrounded by support cells in Ang1-overexpressing tumor xenografts, tumor angiogenesis may be functionally inhibited. VEGF-induced microvascular hyperpermeability represents one of the earliest steps in the angiogenic cascade and provides a matrix that facilitates in-growth of blood vessels[43] and tumor progression.[38] Because Ang1 inhibits vascular permeability[44] it is conceivable that Ang1 overexpression also resulted in a less submissive matrix for tumor growth and progression. Importantly, VEGF mRNA and protein expression in Ang1-overexpressing tumors were comparable to vector control-transfected tumors. Moreover, expression and phosphorylation of VEGFR-2 were also comparable in all tumors studied, suggesting that tumor growth retardation was not mediated by modulation of VEGF activity.

Overexpression of Ang2 did not modulate Tie2 phosphorylation and accordingly failed to affect tumor growth and angiogenesis. It has recently been reported that overexpression of Ang2 in colon and hepatocellular cancer resulted in enhanced tumor growth.[18,19] This may be explained by the reported expression of endogenous Ang1 in those tumor cells, in contrast to A431 cells that do not express any detectable levels of Ang1. It is therefore conceivable that in the presence of tumor cell-derived Ang1, overexpression of Ang2 may promote tumor growth by blocking the growth inhibitory Ang1 signal. This is in agreement with our findings that the inhibitory effect of Ang1 on tumor growth could be partially rescued by Ang2. Moreover, our results indicate that the biological effects of Ang1 and Ang2 are critically dependent on their relative expression levels and consequently on the phosphorylation status of the Tie2 receptor. Therefore, correlation of intratumoral angiopoietin expression with Tie2 phosphorylation is essential to assess the distinct roles of Ang1 *versus* Ang2 expression for tumor growth and angiogenesis. Several lines of evidence strongly indicate that the observed effects of transfected Ang1 and Ang2 on tumor growth were not because of direct effects on tumor cells, because A431 cells do not express the Tie2 receptor and no differences in the growth rate, colony forming ability, or apoptosis rate were observed between the different transfected clones *in vitro*.

In summary, we have identified the up-regulation of Ang2 expression as an early event during multistep carcinogenesis. Moreover, we provide evidence for an inhibitory role of Ang1-mediated Tie2 receptor activation in SCC growth. Finally, our findings suggest an important function of the maturation status of the tumor vasculature in tumor progression.

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