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Cloning and Characterization of Angiocidin, a Tumor Cell Binding Protein for Thrombospondin-1

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Abstract Thrombospondin-1 (TSP-1) is a matrix protein that has been implicated in mechanisms of tumor progression. Our laboratory previously showed that the CSVTCG (cys-ser-val-thr-cys-gly) sequence of TSP-1 functioned as a tumor cell adhesion domain and CSVTCG peptides as well as an anti-peptide antibody possessed anti-metastatic activity in a murine model of lung metastasis. In a subsequent study, a putative TSP-1 binding protein from lung carcinoma was isolated by CSVTCG-peptide affinity chromatography. In this study, we present the full-length cDNA of this binding protein isolated from a prostate cancer cell (PC3-NI) cDNA library. The purified recombinant protein, termed angiocidin, is a potent inhibitor of tumor growth of Lewis Lung carcinoma in vivo and tumor invasion and angiogenesis in vitro. In addition, the recombinant protein inhibits tumor and endothelial cell proliferation and induces apoptosis. The activity of angiocidin both in vivo and in vitro is partially dependent on its TSP-1 binding activity, since an angiocidin deletion mutant missing a high affinity-binding site for TSP-1 failed to inhibit tumor growth in vivo and was less active in its anti-tumor and anti-angiogenic activities in vitro. These results suggest that the anti-tumor activity of TSP-1 reported in many studies may be mediated in part by binding proteins such as angiocidin. Such proteins may function as tumor-suppressor proteins, which limit the growth of tumors by inhibiting angiogenesis and cell matrix interaction. J. Cell. Biochem. 92: 125–146, 2004.

Key words: angiogenesis; metastasis; thrombospondin-1; apoptosis; endothelial cells

The extracellular matrix has long been recognized as an important molecular mediator of tumor progression and metastasis. Such extracellular molecules as proteoglycans, fibronectin, and laminin provide vascular attachment sites for metastasizing tumor cells as well as providing a suitable environment for cell proliferation in the interstial tissue [Ruoslahti, 1996]. Detailed knowledge of the tumor cell binding domains of these matrix molecules has provided a unique opportunity to design peptide antagonists that have been shown to prevent tumor cell implantation in animal models of tumor cell metastasis. For example, synthetic fibronectin peptides and laminin peptides based on the tumor cell adhesion domains RGD for fibronectin [Ruoslahti and Pierschbacher, 1987] and YIGSR for laminin [Barsky et al., 1984] have been shown to block lung colonization of a number of syngeneic mouse tumors. The important role of the extracellular matrix in tumor progression is further exemplified by the findings that fragments of type IV and XIII collagen

Abbreviations used: BSA, bovine serum albumin; BAE, bovine aortic endothelial; CAM, chorioallantoic membrane; CSVTCG, cysteine serine valine threonine, cysteine glycine; cDNA, complimentary deoxynucleotides; *E. coli*, *Escherichia coli*; GST, glutathione S-transferase; HUVE, human umbilical vein endothelial; IgG, immunoglobulin G; PBS, phosphate buffered saline; PCR, polymerase chain reaction; NC, nitrocellulose; SDS–PAGE, sodium doceyl polyacrylamide gel electrophoresis; TBS, tris buffered saline; TSP-1, thrombospondin-1.

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such as tumstatin [Yohei Maeshima et al., 2001] and endostatin [O'Reilly et al., 1997], respectively possess potent anti-tumor activity. Like fibronectin, laminin, and collagen, thrombospondin-1 (TSP-1), a cell adhesive protein and matrix molecule associated with the vascular basement membrane [Arbeille et al., 1991], is present in tumor stroma [Wong et al., 1992; Tuszynski and Nicosia, 1994; Qian et al., 2001], and mediates cell-to-cell and cell-to-matrix interactions important in tumor progression [Bornstein, 2000]. In addition, TSP-1 is secreted by platelets [Lawler et al., 1978].

TSP-1, the most abundant member of the five member thrombospondin gene family, is composed of three identical disulfide-linked chains each consisting of 1,152 amino acids (MW 145,000), and each polypeptide chain is composed primarily of domains consisting of repeating homologous amino acid sequences [Lawler and Hynes, 1986]. These polypeptides contain many sequence dependent cell recognition domains [Sargiannidou et al., 2001]. To identify which TSP-1 sequences mediated the adhesive interactions that promote tumor cell implantation during metastasis, we theorized that such or similar sequences might be functioning during the liver implantation of the malarial parasite. We hypothesized that implantation of this parasite occurred through an adhesive mechanism similar to that of TSP-mediated arrest of metastatic tumor cells. In fact, TSP-1 and the malarial circumsporozoite protein, which is believed to mediate the adhesive activity of the malarial parasite, share sequence homologies [Kobayashi et al., 1986]. The most significant homology is based around the consensus sequence WSPCSVTCG (tryp-ser-pro-cys-ser-val-thr-cys-gly), which is present in three homologous copies in TSP-1, six copies in properdin, and one copy each in all of the malarial circumsporozoite proteins. Therefore, to test whether these TSP-1 sequences played any role in tumor cell metastasis, the cell adhesive activities of synthetic peptides corresponding to these sequences were evaluated in cell adhesion assays, platelet aggregation, and tumor cell lung colonization [Tuszynski et al., 1992a]. We found that a number of peptides homologous to CSVTCG promoted the adhesion of a variety of normal and tumor cells and inhibited platelet aggregation and tumor cell metastasis, whereas control peptides had no effect. Our results further demonstrated that these peptides inhibited tumor lung metastases presumably by competing with endogenous TSP-1 for TSP-1 tumor cell receptor sites. This conclusion was further supported by the observation that anti-peptide antibodies, which specifically recognized TSP-1, inhibited TSP-dependent cell adhesion, platelet aggregation, and tumor cell metastasis, whereas control IgG had no effect [Tuszynski et al., 1992a]. These results suggested that CSVTCG-related peptides present in the type 1 repeat region of TSP-1 functioned in the adhesive interactions of TSP-1 that mediate platelet aggregation and tumor cell metastasis.

In 1993, we isolated a protein from lung carcinoma specific for the CSVTCG residues in the type 1 repeats of TSP-1 by CSVTCG-Sepharose chromatography [Tuszynski et al., 1993]. The CSVTCG binding protein was cell surface exposed on lung carcinoma cells. Anti-binding protein IgG inhibited lung carcinoma cell spreading and adhesion on TSP-1 but not on fibronectin and laminin. The immunohistochemical localization of this protein showed selective expression on invasive cancer cells and capillary endothelial cells [Arnoletti et al., 1994; Tuszynski and Nicosia, 1994; Roth et al., 1997; Wakiyama et al., 2001]. These results suggested that the protein may function in mechanisms of metastasis and angiogenesis.

In this report, we present the full-length cDNA of the CSVTCG binding protein. The purified recombinant protein binds TSP-1 with high affinity and possesses potent anti-tumor activity and anti-angiogenesis activity in vivo and in vitro. A deletion mutant missing a high affinity binding domain for TSP-1 showed no anti-tumor activity in vivo or anti-angiogenesis in vitro. These data suggest that TSP-1 binding proteins such as angiocidin may significantly modulate the activity of TSP-1 in tumor progression.

MATERIALS AND METHODS

Affinity Sensor Assay

A 2 μg quantity of TSP-1 was coupled through its amino groups to carboxyl groups on the affinity sensor cuvette surface. Unreacted groups on the cuvette surface were then blocked with ethanolamine and albumin. Peptides and proteins were added to the cuvette. The binding interaction was monitored by the instrument and constants were calculated using

the Affinity Sensor Software (IAsys, Cambridge, UK). Peptide 1-2, mutated receptor, or full-length receptor in PBS buffer, pH 7, showed saturable binding and the binding could be partially dissociated with buffer or completely dissociated with HCl.

Angiogenesis Assay

Matrigel angiogenesis assay was performed as previously described [Grant et al., 1989]. Briefly, HUVE cells were plated in full-media in 96 well microtiter plates coated with Matrigel in the presence of increasing concentrations of angiocidin. Cells were cultured overnight and endothelial networks were visualized by phase microscopy. The chick CAM assay was performed as previously described [Colman et al., 2000]. Briefly, 8 mm diameter filter disks of number 1 Whatman filter paper were soaked in 3 mg/ml cortisone acetate in a solution of 95% ethanol. Sterile filter disks soaked with bFGF served as the positive control. In a second area of the CAM, filter disks were absorbed with bFGF and treated with 200 μg/ml solution of angiocidin or M1. The CAMs were incubated for 72 h at 37°C and maintained at 55% humidity. At least five CAMs were used per treatment group. Each experiment was performed at least three times. The anti-angiogenic activity of angiocidin and M1 was assessed by counting the number of radial vessels that developed.

Annexin V Binding

Apoptosis as measured by annexin V binding was performed as described in the annexin V binding kit (BD Biosciences, Palo Alto, CA). Briefly, HUVE cells in full media were plated in six well plates coated with or without 40 μg/ml of angiocidin and cultured overnight. Cells were then treated with fluorescent annexin V according to the instructions provided in the kit. Cells were photographed under phase contrast and fluorescent microscopy.

Antibodies and Reagents

Monoclonal and polyclonal antibodies against angiocidin were prepared from purified recombinant protein (Covance, Denver, PA).

Boyden Chamber Invasion Assay

Cell invasion was performed using a modified Boyden chamber. Polycarbonate filters, 8 μm pore size (Millicell, Millipore Corporation, Bedford, MA), were coated with 100 μg type I collagen (1 mg/ml 60% ethyl alcohol) and dried overnight at 25°C. Blind-well Boyden chambers were filled with 600 μl of DMEM with 2% horse serum in the lower compartment, and the coated filters were mounted in the chamber. Approximately 50,000 cells (tested to be greater than 95% viable) suspended in 200 μl of DMEM with 0.1% BSA were placed in the upper chamber of the apparatus and allowed to settle onto the collagen-coated membrane. Varying concentrations of angiocidin and M1 (0–100 μg/ml) were placed in the upper compartment and any neutralizing antibodies were also placed in the upper chamber. After an incubation period of 16 h at 37°C, the cells on the upper surface of the filter were removed with a cotton swab. The filters were then fixed in 2.5% glutaraldehyde solution and stained with 0.5% crystal violet solution. Invasive cells adhering to the undersurface of the filter were counted using a phase contrast microscope (400×). The data was expressed as the summation of the total number of invasive tumor cells.

Cell Culture

Lewis lung carcinoma and MDA-MB-231 breast carcinoma were grown in DMEM medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (FBS), 50 U/ml of penicillin, 50 μg/ml of streptomycin, and 50 μg/ml of gentamicin sulfate and kept in 5% CO_2 at 37°C. Bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial (HUVE) cells were cultured in EGM and EGM-2 media, respectively (Cambrex Corporation, East Rutherford, NJ).

Cell Staining

HUVE cells were grown on tissue culture glass slides and fixed with 90% cold ethanol. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide treatment for 10 min. Cells were subsequently incubated for 1 h with 5% normal horse serum and then cells were incubated with the primary mouse polyclonal anti-angiocidin antibody serum made against recombinant angiocidin, diluted 1:1,000 in PBS containing 0.1% BSA, at 4°C overnight. As a negative control, mouse serum was used in every experiment. The fluorescein anti-mouse IgG (Vector Laboratories, Burlingame, CA) was used as a secondary antibody. Then the cells were incubated with a monoclonal antibody to TSP-1 (#4 from NeoMarkers, Fremont, CA) at a

concentration of 4 μg/ml. The secondary antibody used was R-*Phycoerythrin* anti-mouse IgG (Sigma-Aldridge, Saint Louis, MO). Cells were examined under a fluorescent microscope and photographed.

Colorimetric TSP-1 Binding Assay

Angiocidin was immobilized on microtiter plates by overnight incubation of wells with 200 μl of a 40 μg/ml angiocidin solution in PBS. Plates were blocked with 1% BSA in Tris-buffered saline (TBS) for 2 h. TSP-1 was biotinylated as described below. Unreacted biotin was removed by gel filtration. The binding buffer consisted of TBS, pH 7.2 containing 0.05% Tween-20 and 0.1% BSA to minimize non-specific binding. All reagents are dissolved in this buffer. A 100 μl aliquot of 5.7 nM solution of biotinylated TSP-1 in binding buffer was added to each derivatized well and incubated for 2 h with shaking. Wells were washed with 100 μl of binding buffer for 2 min with shaking and buffer aspirated and the washing procedure repeated twice. Wells were incubated with 100 μl of neutra-avidin (purchased from Pierce, Rockford, IL) for 30 min with shaking. Wells were washed five times with shaking. Wells were developed for 10–20 min with 100 μl of the chromogen from Pierce containing peroxide and 3,3′, 5,5′tetramethyl benzidine. The reaction was stopped with 50 μl of 0.5 normal sulfuric acid and the absorbance was read at 450 nm in a microplate reader.

Construction, Expression, and Purification of His-Tagged Recombinant Angiocidin

Full-length cDNA cloned in the pBK-CMV vector was used as a template to generate a PCR product that contained the correct restriction sites, Bgl II and EcoR I, enabling the DNA to be ligated into the His tag vector pTrcHisA with BamH I and EcoR I sites. This expression construct was sequenced to verify PCR fidelity. PTrcHisA vector allows expression of foreign proteins in *E. coli* as fusions to the N terminus of six histidines, which can be affinity purified under non-denaturing conditions. The vector alone or expression construct was used to transform *E. coli* BL21 cells.

Expression and purification of angiocidin was prepared using a Qiagen kit and protocol (Qiagen, Valencia, CA). Briefly, overnight cultures of transformed *E. coli* were diluted in fresh medium and grown for a further 3 h at 37°C to $OD_{600} = 0.5–0.8$. Protein expression was induced with 1 mM isopropyl-β-D thiogalactopyranoside, and after a further 4 h at 37°C, the cells were pelleted and resuspended in lysis buffer provided by Qiagen containing lysozyme (1 mg/ml). Cells were then sonicated and centrifuged at 12,000*g* for 10 min; the supernatant containing the expressed fusion proteins was purified by passing the lysates through Ni-NTA resin (Qiagen). The fusion proteins were finally eluted by elution buffer containing a high concentration of imidazole. Endotoxin was completely removed from recombinant proteins by Triton X114 phase separation as previously described [Aida and Pabst, 1990].

Expression of Angiocidin Deletion Mutants

Sense and antisense oligonucleotides corresponding to angiocidin-sequence were used in PCR amplification of a set of overlapping products with the previously isolated angiocidin cDNA clone. The three fragments were obtained by using primer pairs a/b, c/d, and e/f (primers: a, GGGAGATCTATGGTGTTGGAAAGCACT; b,CCGGAATTCCTTGAGGCGTTTAGCCAG; c,CGCGGATCCGATCTGGTGAAACTGGCT; d,CGGAATTCTTCACCTTCAGTCCCAGT; e,GGAAGATCTATTGCTACGACTGGGACT; f,GGGGAATTCTCACTTCTTGTCTTCCTC), which have Bgl II and EcoR I sites compatible with BamH I and EcoR I sites in the pGEX-2T expression vector (Pharmacia, Peapack, NJ). The three resulting PCR-products were digested with Bgl II and EcoR I (Promega, Madison, WI), purified with PCR purification kits (Promega), ligated to the Bam HI and Eco RI sites of pGEX-2T, and transformed into *E. coli*. The inserted DNAs were verified by the dideoxy chain termination sequencing.

Expression and Purification of Fusion Proteins

Glutathione S-transferase (GST) fusion proteins were expressed in *E. coli* after induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested 4 h after induction. After sonication and centrifugation, the supernatant containing the expressed fusion proteins were purified by passing the lysate through glutathione-agarose (Pharmacia). The control protein, GST, was also expressed using an empty vector.

Expression and Purification of M1

A set of primers (a, GGGAGATCTATGGTGTTGGAAAGCACT; b, ATCTTGGTGATCTTGCCCTTGGG; c, TCACCAAGATGCGCATCATTGC; d, GGGGAATTCTCACTTCTTGTCTTCCTC) was used to delete 20 amino acids from the full-length protein. Two fragments were made by PCR using primer a and b, and primer c and d, respectively. Then these two fragments were joined by PCR by using primer a and d. The rest of the cloning procedure is the same as described above for full-length angiocidin.

Immunohistochemical Localization of Angiocidin and Microvessel Density Measurements

Tumor tissue as well as samples of various organs were embedded in paraffin, sectioned, and stained with a mouse anti-his tag monoclonal antibody (Clonetech, Palo Alto, CA) and factor eight antibody (R&D Systems, Minneapolis, MN) using a protocol provided by the Vectastain ABC immunoperoxidase staining kit (Vector Laboratories) and as previously performed in our laboratory [Tuszynski and Nicosia, 1994]. In addition, tissue arrays were stained using a kit from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoprecipitation

A T75 flask (7–10 million cells) of HUVE cells were solubilized in 200 μl of M-Per (Pierce) buffer containing protease inhibitors. Half of the lysate was treated with 10 μl of goat-anti-TSP-1 and the other half treated with 10 μl non-immune serum. Lysates were incubated for one hour at 37°C followed by addition of a 20 μl of a 50–50 slurry of protein A agarose suspended in PBS and incubated for an additional hour. Protein A pellets were washed in Pierce binding buffer, and extracted with 100 μl of SDS-sample buffer containing reducer. Immunoprecipitates were then separated by SDS–PAGE, electroblotted for TSP-1 using 2 μg/ml mouse anti-TSP-1 monoclonal (#4 from NeoMarkers), membranes stripped, and then probed for angiocidin using 5 μg/ml mouse monoclonal anti-angiocidin antibody.

Isolation of Angiocidin cDNA

Polyclonal antisera against angiocidin isolated from A549 human lung carcinoma [Tuszynski et al., 1993] was used to screen a lambda ZAP-Express (Stratagene, La Jolla, CA) prostate cancer cell (PC3-NI) cDNA library kindly provided by Dr. Min Wang (Drexel College of Medicine, Phila, PA). For screening of the library, phages were plated at 3×10^4 plaque-forming units per 150-mm dish on *E. coli*. Rabbit antiserum against angiocidin was absorbed with 0.5 volumes of *E. coli* Y1090 lysate for 4 h at 23°C and diluted to a ratio of 1:1,000; screening was performed according to the PicoBlue Immunostaining kit (Stratagene). Alkaline-phosphatase-conjugated goat anti-rabbit antibodies were used as secondary antibodies. Approximately 200,000 plaques were screened and four positive plaques were picked, eluted into phage suspension medium, re-plated, and screened until all plaques tested positive.

Phagemids from phage were transferred to XL-1 Blue bacteria using the Exassist Interference-Resistant Helper Phage protocol. Plasmid DNA was purified using the Wizard plus miniprep (Promega) and partially sequenced using the T7/T3 primer set.

DNA from Plasmid pBK-CMV vector was used as a template for sequencing of the insert by dideoxy chain termination method with Sequenase version 2.0 (US Biochemical Amersham, Cleveland, OH). Initial forward and reverse primers copying regions of pBK-CMV immediately upstream and downstream of inserted DNA were T7/T3 obtained from Promega. Subsequent primers were synthesized on the basis of sequence information obtained. Sequence alignments and protein analysis was made using the BioNavigator program (Entigen Corporation, Sunnyvale, CA).

Ligand Blots

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of purified proteins and prestained molecular weight standards were performed in a 12% acrylamide mini-slab gel. Proteins were then either stained with Coomassie Brilliant Blue or transferred electrophoretically to nitrocellulose (NC) membrane for a ligand blot analysis. TSP-1 was biotinylated using the Pierce protein biotinylation protocol (EZ-Link Sulfo-NHS-LC-Biotin, Pierce Chemical Co., Rockfort, IL). Bacterial lysate expression receptor fragments and empty vector controls were separated by SDS–PAGE and electroblotted on to NC paper. NC membranes were incubated in PBS/3% NP40 for 30 min, washed, and blocked with PBS/3% BSA for 2 h,

incubated with PBS/0.1% Tween 20 for 10 min, and then incubated with TSP-1 or CSVTCG-biotin in PBS/1% BSA/0.1% Tween20 at 4°C overnight. Streptavidin–peroxidase was added at a dilution of 1:5,000 in PBS/1% BSA/0.1% Tween 20 for 1 h. The membrane was developed using the chemiluminescence (ECL) system (Amersham, Arlington Heights, IL).

Synthesis of Peptides

The short overlapping peptides (1–4, MVLESTMVCVDNSEYMRNGDFLPTR; 1–5, FLPTR LQAQQDAVNIVCHSKTRSNP; 1–6, TRSNP ENNVGLITLANDCEVLTTLT; 1–1, LTTLTPDTGRILSKLHTVQPKGKIT; 1–2, KGKITFCTGIRVAHLALKHRQGKNH; 1–3, QGKNHKMRIIAFVGSPVEDN EKDLV; 2–1, EKDLV KLAKRLKKEKVNVDIINFGE) spanning fragment 1 were synthesized by Alpha Diagnostic International (San Antonio, TX). The peptides were greater than 70% pure.

Thrombospondin-1 Purification

TSP-1 was purified from Ca^{2+} ionophore A23187-activated platelets in our laboratory as previously described [Tuszynski et al., 1985]. Purity was assessed by SDS–PAGE using Coomassie blue or silver staining.

Tumor Growth

For one experiment, 25 BDF1 (C57B1/6 × DBA2) mice were acclimated to the animal facility for 1 week and divided into five groups consisting of five animals per group. Animals were then injected subcutaneously in the hind flank with 0.1 ml of 1.0×10^6 Lewis Lung carcinoma suspended in PBS. Seven to nine days after tumor inoculation, animals were treated every other day with iv injections of the following: group 1—PBS (vehicle control), group 2—angiocidin at 2.5 mg/kg, group 3—angiocidin at 1.0 mg/kg, and group 4 at 0.40 mg/kg. Treatments were continued every other day for 5–7 days. In some experiments, animals were treated with daily ip injection of 1 mg/kg. Tumor dimensions were measured every day with a dial-caliper and the tumor volume approximated as $\text{width}^2 \times \text{length} \times 0.52$. Two days after the last treatment, animals were euthanized and tumors examined by histology and stained for his tagged angiocidin. Experiments were repeated to insure reproducibility.

Viability Assay

We evaluated the effect of angiocidin on cell viability and growth using the Alamar blue assay. In this assay, 10 μl Alamar blue, a metabolizable dye, was added to 100 μl of media in a 96 well plate containing 15,000 cells per well. After 2–3 h, the number of viable cells in the plate was proportional to the difference in absorbance at 562 and 595 nm as read in a microtiter plate reader. Dose-dependent effects of angiocidin and M1 in the range of 10–200 μg/ml were measured.

Western Blotting

Recombinant proteins were fractionated on 8–12% gradient SDS–PAGE and then transferred to NC membrane using a Biorad electrophoresis system. Nonspecific sites of the membranes were blocked with 5% skim milk in TBS containing 0.1% Tween 20 (TBS-T) for 1 h. The immunoblots were incubated with primary antibodies diluted in TBS-T for 1 h. After washing, the immunoblots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The immunoreactivity was detected using the ECL system (Amersham).

RESULTS

Molecular Cloning and Sequence Analysis of Angiocidin

A rabbit polyclonal antibody prepared against the lung tumor CSVTCG binding protein was used for screening a cDNA library prepared from human prostate cancer. Four positive clones expressing immunoreactive protein were isolated among 200,000 plaques. These clones were rescued from λ phage and recloned into the Bam HI and Eco RI sites of vector pBK-CMV. Sequencing analysis showed that these four clones belonged to the same gene. A database search of the full-length cDNA sequence revealed an identical sequence to a protein present in mouse and rat and a high homology to two human proteins that differed by lacking only three amino acids (GER) near the carboxyl terminus. The total reconstructed cDNA contained 1,309 base pairs followed by a polyA tail with an open reading frame of 1,146 base pairs.

Using the primer pair spanning the whole sequence, we confirmed the full-length coding sequence by using RT-PCR amplification of tumor cell and fibroblast RNA. The open read-

ing frame encoded 382 amino acids with a calculated molecular mass of 41 kDa and a calculated PI of 4.9. It is also homologous to *Arabidopsis* MBP1 [van Nocker et al., 1996] as well as to a 50-kDa 26S protease subunit form *Drosophila* [Szlanka et al., 2003] and *Saccharomyces* [Kominami et al., 1997]. All show a high degree of similarity over the first 190 amino acids (50–69% identity). This relatively well-conserved N-terminal portion is followed by a glycine-rich region in each protein (residues 190–205 for *Drosophila* and human and residues 190–215 for the yeast and plant sequences). A hydropathy plot of the protein revealed a 40 amino acid hydrophobic sequence spanning residues 160–200 flanked by two hydrophilic sequences. Figure 1 shows the multiple alignments of these proteins.

Expression of Recombinant Angiocidin

The full-length cDNA was ligated into pTricHis A vector so that the open reading frame was in frame with the 6-histidine tag. The constructs were transformed into *E. Coli*, and expression of the fusion protein was induced with IPTG. The purified protein was recovered from Ni-NTA resin and the total protein from bacterial extracts was subjected to SDS–PAGE and Western blotting. Figure 2A shows the Coomassie Blue stained SDS–PAGE gel. The purified protein has a molecular weight of 60 kDa, which is higher than the estimated molecular mass from the amino acid sequence. These bands were recognized by Western blot using our polyclonal receptor antibody prepared against receptor purified from tumor extracts (Fig. 2B). This strong reaction indicated that this recombinant protein has the same immuno-reactivity as the CSVTCG-specific receptor previously purified by column chromatography [Tuszynski et al., 1993].

Expression Levels of Angiocidin in Different Tumor Tissues

We previously investigated the pattern of expression of the CSVTCG-specific receptor in breast carcinoma [Tuszynski and Nicosia, 1994], head and neck carcinoma [Arnoletti et al., 1994], and colon carcinoma [Wakiyama et al., 2001] and found high expression relative to normal tissue. Using a monoclonal antibody prepared against recombinant angiocidin and tissue arrays containing ten cases each of advanced stage cancer listed in Figure 3 as well as their corresponding normal tissues, we examined the expression of angiocidin by immunohistochemistry. We found that angiocidin was highly expressed in the tumor epithelium with relative low expression in the epithelium of the corresponding normal tissue (Fig. 3).

TSP-1 Binding Activity of Recombinant Protein

The binding activity of the recombinant protein produced in *E. coli* was evaluated by TSP-1 ligand blot, binding of TSP-1 to immobilized angiocidin, and binding of angiocidin to immobilized TSP-1 using affinity sensor analysis. The latter two methods measured binding under non-denaturing conditions. For ligand blot analysis, both TSP-1 and the peptide CSVTCG were biotinylated using the Pierce protein biotinylation protocol. Unreacted biotin was removed by dialysis. Bacterial lysates expressing receptor and empty vector controls were separated by SDS–PAGE and electroblotted on to NC paper. Biotin labeled TSP-1 and CSVTCG were used to bind to angiocidin. We found that both TSP-1 and CSVTCG peptide bound angiocidin (Fig. 4A), while whole bacterial extract not expressing angiocidin (lanes 1, 3, 5), bound no TSP-1 or CSVTCG.

Angiocidin immobilized on microtiter plates bound biotinylated TSP-1 saturably with a K_D of 47 nM (Fig. 4B). Binding was considered specific since biotinylated TSP-1 did not bind appreciably or saturably to BSA (Fig. 4B) and the binding of biotinylated TSP-1 to angiocidin could be competed with unlabeled TSP-1 (data not shown).

In affinity sensor binding experiments, angiocidin in HEPES buffered saline (pH 7) showed saturable binding and the binding could be partially dissociated with buffer or completely dissociated with HCl (Fig 4C). A dissociation constant of 44 nM was calculated from a plot of the pseudo first order rate constant for association versus the concentration of the angiocidin. Addition of the detergent Tween 20 to the buffer did not alter the binding suggesting that this interaction is specific. Additionally, we were able to inhibit angiocidin binding by at least 50% with a 500 M excess of CSVTCG, whereas a scrambled peptide control had no effect (data not shown).

Angiocidin Binds TSP-1 In Vivo

To investigate whether endogenous angiocidin binds to TSP-1 in vivo, we utilized two

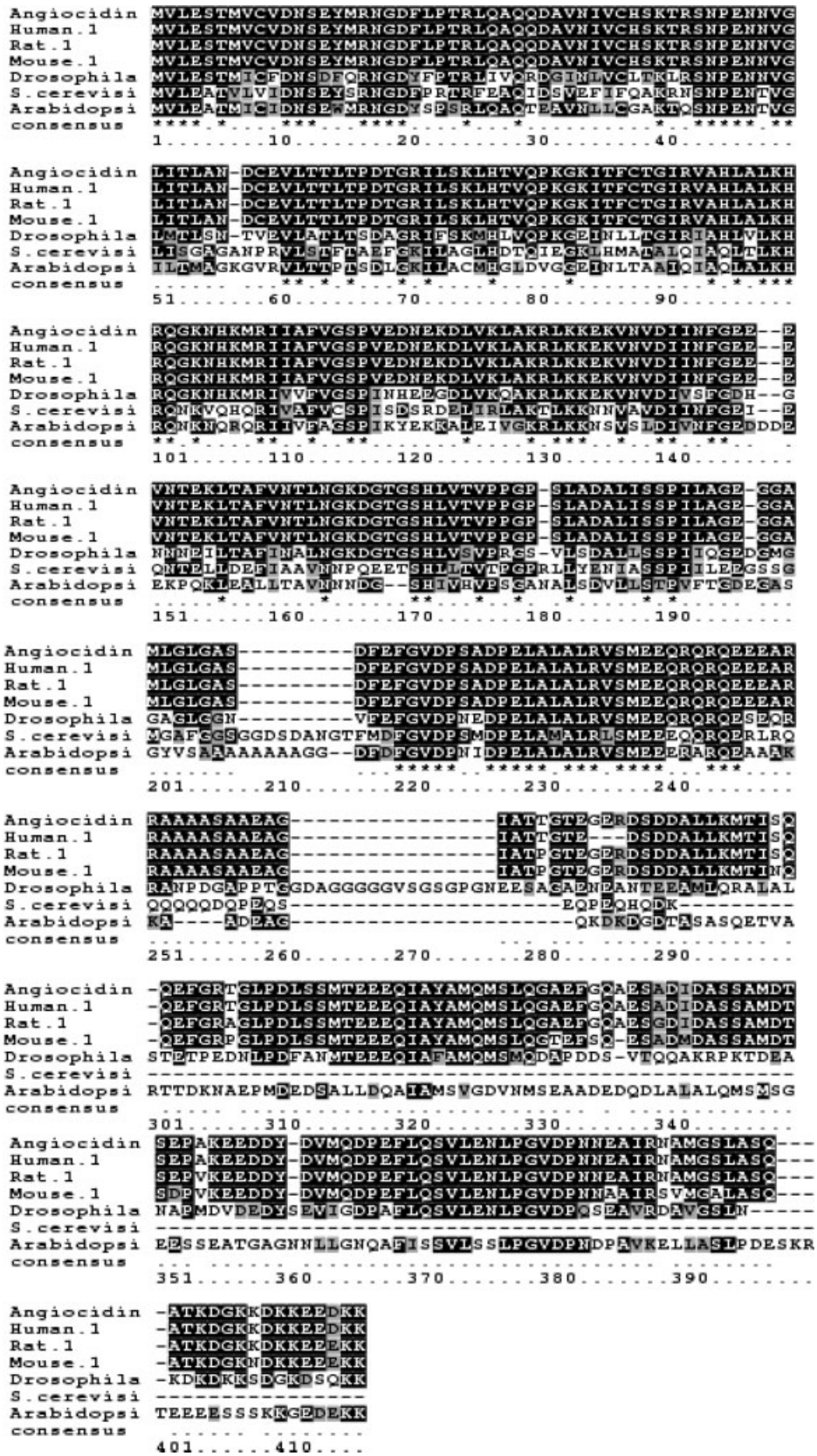

Fig. 1. Comparison of angiocidin sequences from human, rat, mouse, *Drosophila*, *S. cerevisiae*, and *Arabidopsis* proteins. Positions at which a majority of the residues are identical have been denoted by white letters on black.

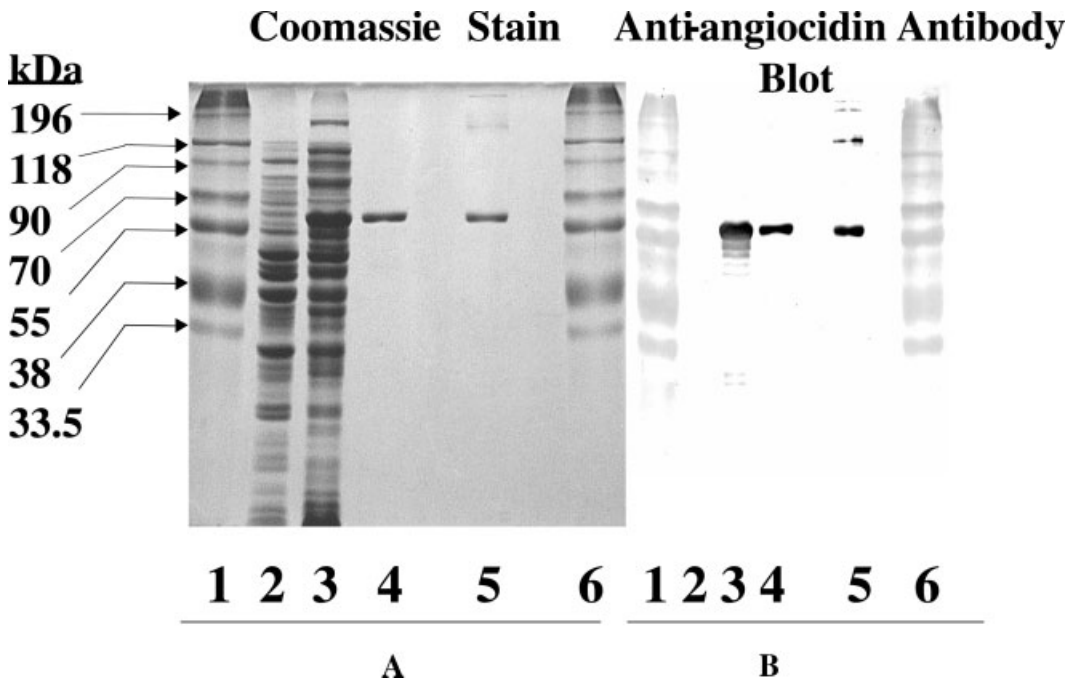

Fig. 2. Expression of purified recombinant angiocidin. Bacterial extracts containing expressed angiocidin, empty vector controls, and purified his-angiocidin were analyzed by SDS–PAGE and blots stained with anti-angiocidin antibody. For Western blotting, membranes were treated with 1:2,000 angiocidin antibody serum in TBS-tween (Tris-buffered saline containing 0.05% Tween 20) for 2 h, washed in TBS-tween, probed for 1 h with 1:15,000 horseradish peroxidase-conjugated anti-rabbit IgG, washed, and then revealed by ECL. **Panel A**: stained gel; **Panel B**: anti-angiocidin antibody blot. 1, prestained MW standards; 2, detergent bacterial extract with no insert; 3, detergent bacterial extract with angiocidin insert; 4, reduced his-tag purified angiocidin; 5, non-reduced his-tag purified angiocidin; 6, prestained MW standards.

approaches. We double labeled immunohistochemically localized TSP-1 and angiocidin in endothelial cells and evaluated whether TSP-1 and angiocidin would co-immunoprecipitate from these cells (Fig. 5). TSP-1 and angiocidin co-localized in the cytoplasm and membrane of HUVE cells grown in culture (Fig. 5A). When TSP-1 immunoprecipitates from HUVE cells were blotted with anti-angiocidin antibody, angiocidin was detected, whereas control IgG immunoprecipitates were negative (Fig. 5B). These data provide strong evidence that TSP-1 and angiocidin associate in vivo.

Angiocidin Induces Cell Apoptosis In Vitro

To begin to investigate the physiological function of angiocidin, recombinant angiocidin was expressed and its activity evaluated in vitro. We first added increasing concentrations of angiocidin to endothelial cells and measured cell viability 24 h later. Angiocidin induced a dose-dependent loss of BAE cell viability when cultured in serum-containing media (Fig. 6A). After 24 h, cells at the highest concentration of angiocidin became rounded and lost metabolic activity. These cells displayed DNA laddering (data not shown) and binding of annexin V characteristic of cell apoptosis (Fig. 6B). The apoptotic activity of angiocidin was also evident when cells were plated on angiocidin-coated plates and could be reversed by anti-angiocidin monoclonal antibody (Fig. 6C). In addition, angiocidin induced tumor cell apoptosis when cells were plated on angiocidin-coated plates for 24–48 h (red bar, Fig. 6C). However, in contrast to endothelial cells, we found that tumor cells plated on collagen and fibronectin were resistant to the apoptotic effects of endogenously added angiocidin. These data provide evidence that angiocidin can mediate apoptosis in vitro.

Angiocidin Inhibits Tumor Cell Invasion and Angiogenesis In Vitro

Since our immunohistochemical studies revealed that angiocidin is not only expressed by tumor cells but is also present in tumors and that we can detect angiocidin in the extracellular matrix of tumor and endothelial cells grown in culture, we evaluated the effect of

Tumors	Stage
Breast	IIA-IIIA,B
Lung	Squamous Cell Carcinoma
Ovarian	Ia-IIIa,c
Bladder	I-IV
Prostate	I-IV
Pancreatic	II-IVA
Liver	II-IIIA,B
Kidney	Renal Cell
Melanoma	Lymph Node Metastasis

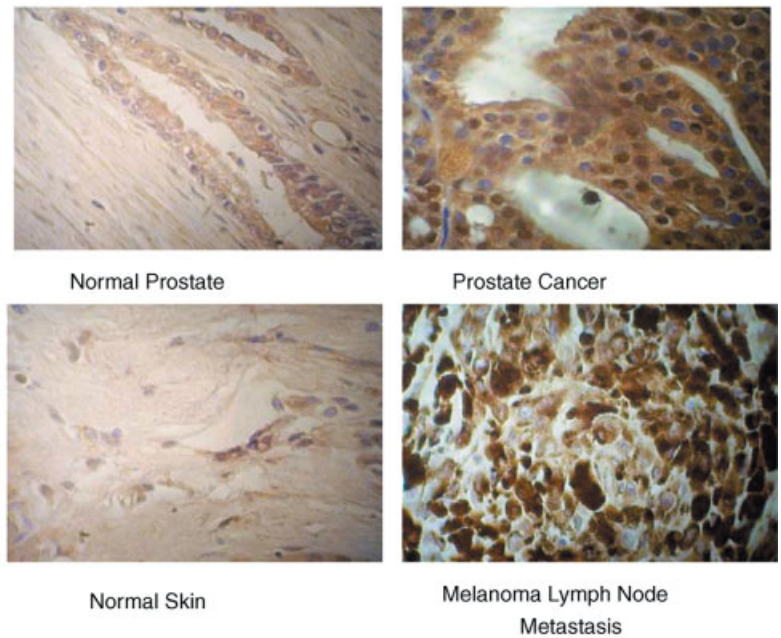

Fig. 3. Human tumors over-expressing angiocidin. Human tissue array slides (Imgenex, San Diego, CA) were immunohistochemically stained with anti-angiocidin monoclonal antibodies using the Santa Cruz immunostaining kit as described by the manufacturer and counterstained with hematoxylin. Photomicrographs were prepared at a magnification of 200×.

exogenously added angiocidin on tumor cell invasion into the collagen, induced by serum containing media. In addition, tube formation inhibition of endothelial cells by exogenously added angiocidin was evaluated in Matrigel (Fig. 7). We found that angiocidin added to the upper chamber of a Boyden chamber coated with type 1 collagen inhibited cell invasion in a dose-dependent manner (Fig. 7A). The invasive chemo-attractant in this experiment was serum-containing media but a similar effect was observed only when TSP-1 was present in serum-free media (data not shown). The inhibitory effect of angiocidin could be reversed with a monoclonal antibody to angiocidin indicating that inhibition was specific (data not shown). Similarly, angiocidin inhibited HUVE cell tube formation on Matrigel in a dose-dependent manner (Fig. 7B). Cells were plated on Matrigel in full endothelial media containing serum. These results indicate that angiocidin can inhibit critical steps in tumor progression that involve cell adhesion and angiogenesis.

Angiocidin Inhibits Tumor Growth In Vivo

Although angiocidin exhibited a number of activities in vitro suggesting that it may play a significant role in regulating tumor growth in vivo, we sought to test the in vivo role of angiocidin in regulating tumor growth. We chose the subcutaneous growth of Lewis lung tumor cells as our in vivo model because our results indicated that Lewis lung tumor cells were inhibited by angiocidin in vitro and that this model produced highly vascularized tumors that respond to anti-angiogenic therapy [O'Reilly et al., 1996]. We found that angiocidin administered systemically by intravenous (iv) injection on the day tumors appeared and then continued every other day for a week inhibited tumor growth by more than 90% at a dose of 2.5 mg/kg (Fig. 8A). The anti-tumor response was dose-dependent and we observed a remarkable 70% inhibition of tumor growth at doses as low as 0.40 mg/kg (Fig. 8B). Histology of the tumors at autopsy revealed that microvessels

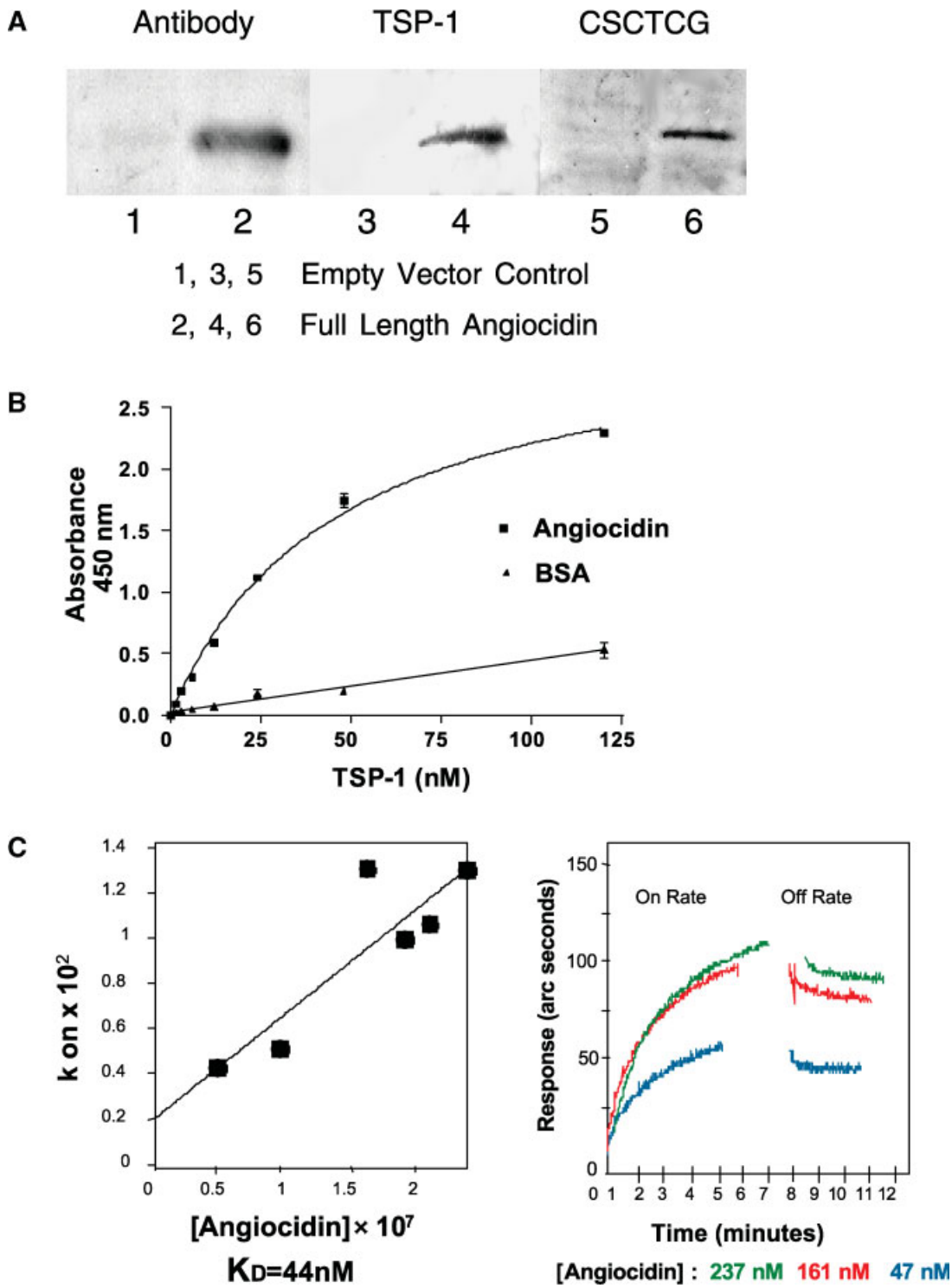

Fig. 4. Binding of recombinant angiocidin to TSP-1. **Panel A**: SDS–PAGE blots of bacterial lysates containing expressed receptor (**lanes 2, 4, 6**) or control lysates containing no expressed angiocidin (**lanes 1, 3, 5**) were either stained with anti-angiocidin antibody (lanes 1, 2), biotinylated TSP-1, (lanes 3, 4), or biotinylated C(Acm)SVTC(Acm)G (lanes 5, 6). **Panel B**: TSP-1 binding was performed as described in Materials and Methods. Immobilized BSA served as control. Curves were drawn from least squares fit of the data using Prizm Graph pad software, San Diego, CA. Each data point is the mean of three replicates and the experiment shown is representative of several determinations. **Panel C**: Angiocidin binding to TSP-1 was performed using affinity sensor technology as described in Materials and Methods. Binding constants were calculated from the binding kinetics as described by the manufacturer (IAsys, Cambridge, UK). The experiment is representative of several determinations.

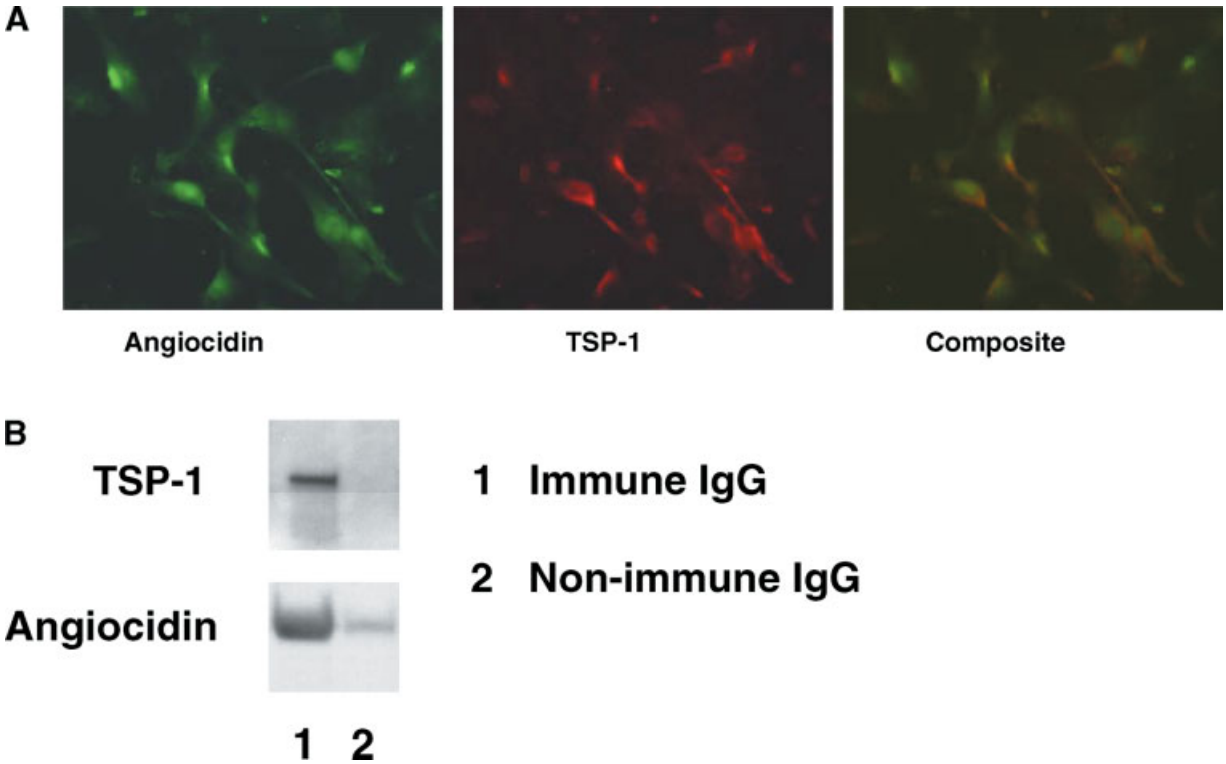

Fig. 5. Angiocidin associates with TSP-1 in endothelial cells. **Panel A**: TSP-1 and angiocidin were co-localized in HUVE cells as described in Materials and Methods. Cells were photographed at 200×. In the panel labeled composite, TSP-1 and angiocidin labeled cells were overlapped. **Panel B**: HUVE cells were immunoprecipitated with anti-TSP-1 antibody or control serum and the immunoprecipitates probed by Western blotting for TSP-1 and angiocidin as described in Materials and Methods.

were absent in angiocidin treated mice and tumor tissue showed rampant necrosis while all of the organs including liver, kidney, intestine, lung, and brain showed no evidence of necrosis (data not shown). These data indicate that angiocidin specifically inhibits tumor growth with no discernible toxic side effects.

Localization of the TSP-1 Binding Domain in Angiocidin

We asked the question whether the anti-tumor activity of angiocidin was dependent on its capacity to interact with TSP-1. To address this question we mapped the TSP-1 binding site on angiocidin. Three recombinant pieces of angiocidin were expressed and probed for TSP-1 binding by TSP-1 ligand blotting (Fig. 9A). We found that the amino terminal fragment possessed the highest TSP-1 binding activity. Overlapping peptides were then synthesized spanning this region (Fig. 9B) and tested for their capacity to inhibit TSP-1 binding to immobilized angiocidin using biotinylated TSP-1 as described in the legend of Figure 4. Peptide 1–2 showed a dose-dependent inhibition of binding while the other peptides showed no inhibition (data not shown). Based on these results, a deletion mutant (M1) lacking the amino acids comprising peptide 1–2 was expressed and

Fig. 6. Angiocidin induces apoptotic cell death of tumor and endothelial cells. **Panel A**: BAE cells plated in 96 well microtiter plates were treated overnight with increasing doses of angiocidin and cell viability determined using Alamar Blue as described in Materials and Methods. **Panel B**: HUVE cells were plated in six well-culture plates coated with and without angiocidin and cultured overnight. Cells were then stained with fluorescent labeled annexin v according to the instructions provided in the annexin V staining kit (BD Biosciences, Palo Alto, CA). Cells were photographed at a magnification of 200×. **Panel C**: Cells plated in 2% serum in 96 well-microtiter plates coated with 2 μg of angiocidin or cells treated with 100 μg/ml angiocidin and plated on plastic were cultured overnight and viability measured using the Alamar Blue assay.

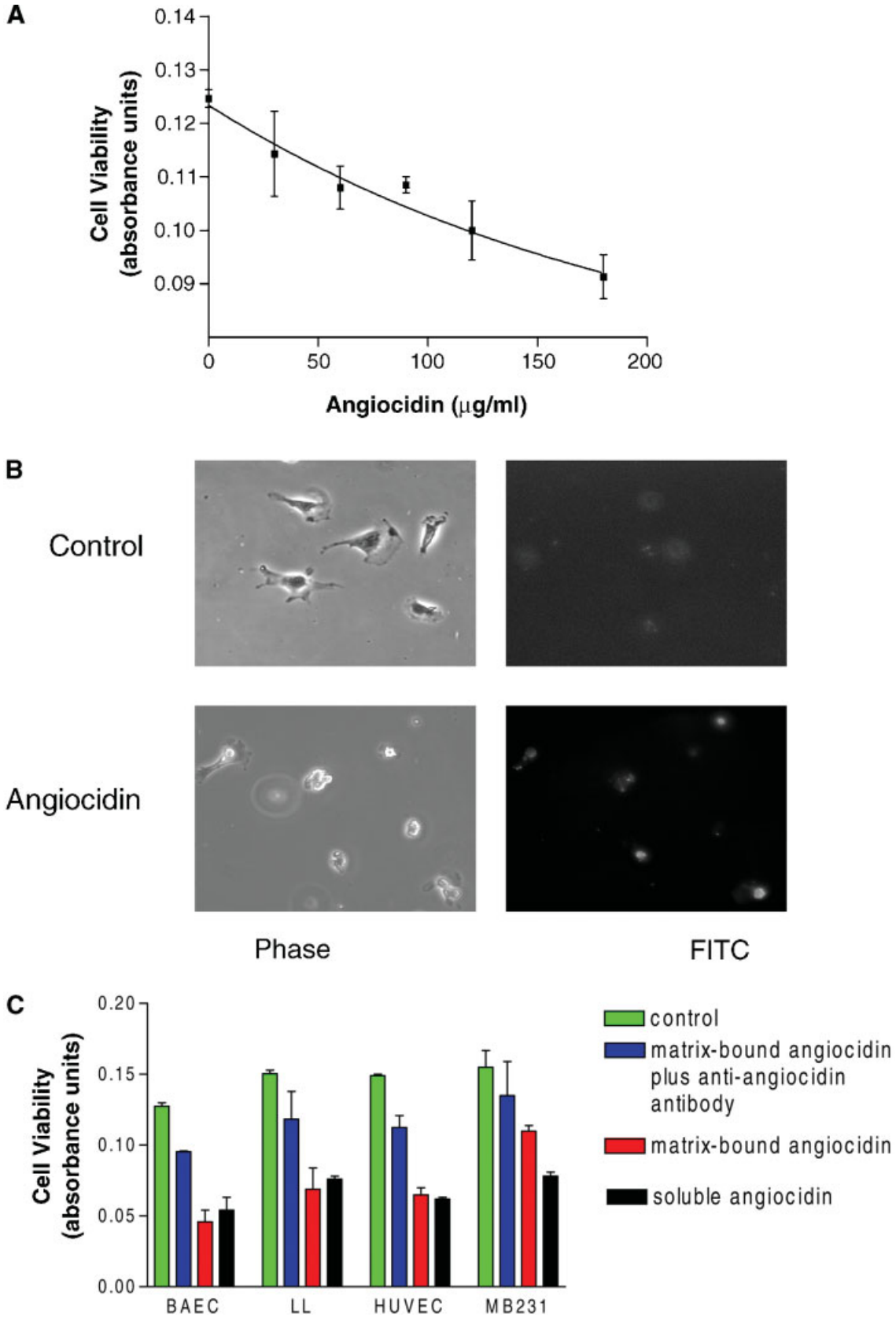
A
0.14
0.13
0.12
0.11
0.10
0.09
Cell Viability (absorbance units)
0
50
100
150
200
Angiocidin (μg/ml)
B
Control
Angiocidin
Phase
FITC
C
0.20
0.15
0.10
0.05
0.00
Cell Viability (absorbance units)
BAEC
LL
HUVEC
MB231
control
matrix-bound angiocidin plus anti-angiocidin antibody
matrix-bound angiocidin
soluble angiocidin

Fig. 6.

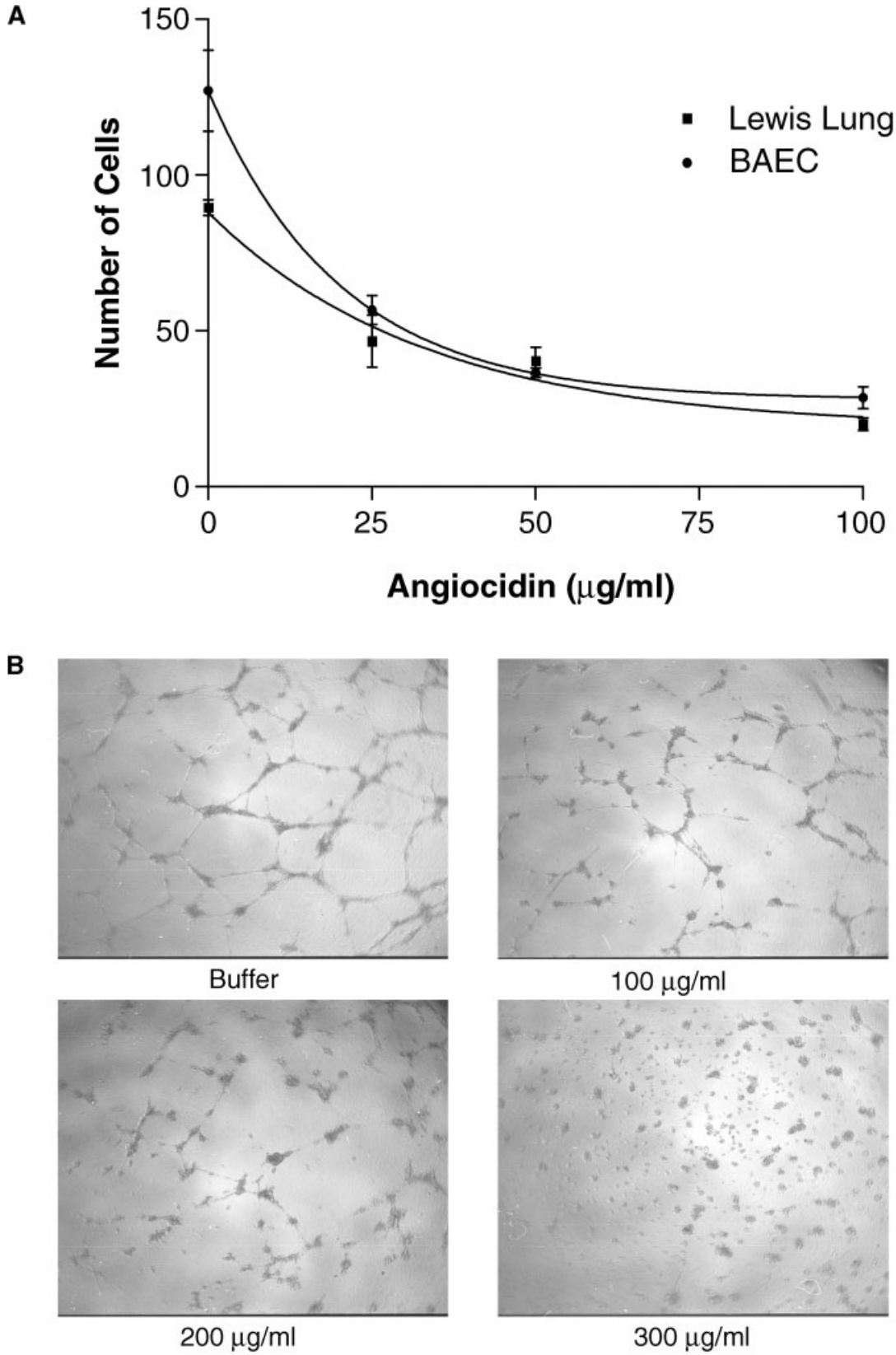
A
150
100
50
0
Number of Cells
Lewis Lung
BAEC
0
25
50
75
100
Angiocidin (μg/ml)
B
Buffer
100 μg/ml
200 μg/ml
300 μg/ml

Fig. 7.

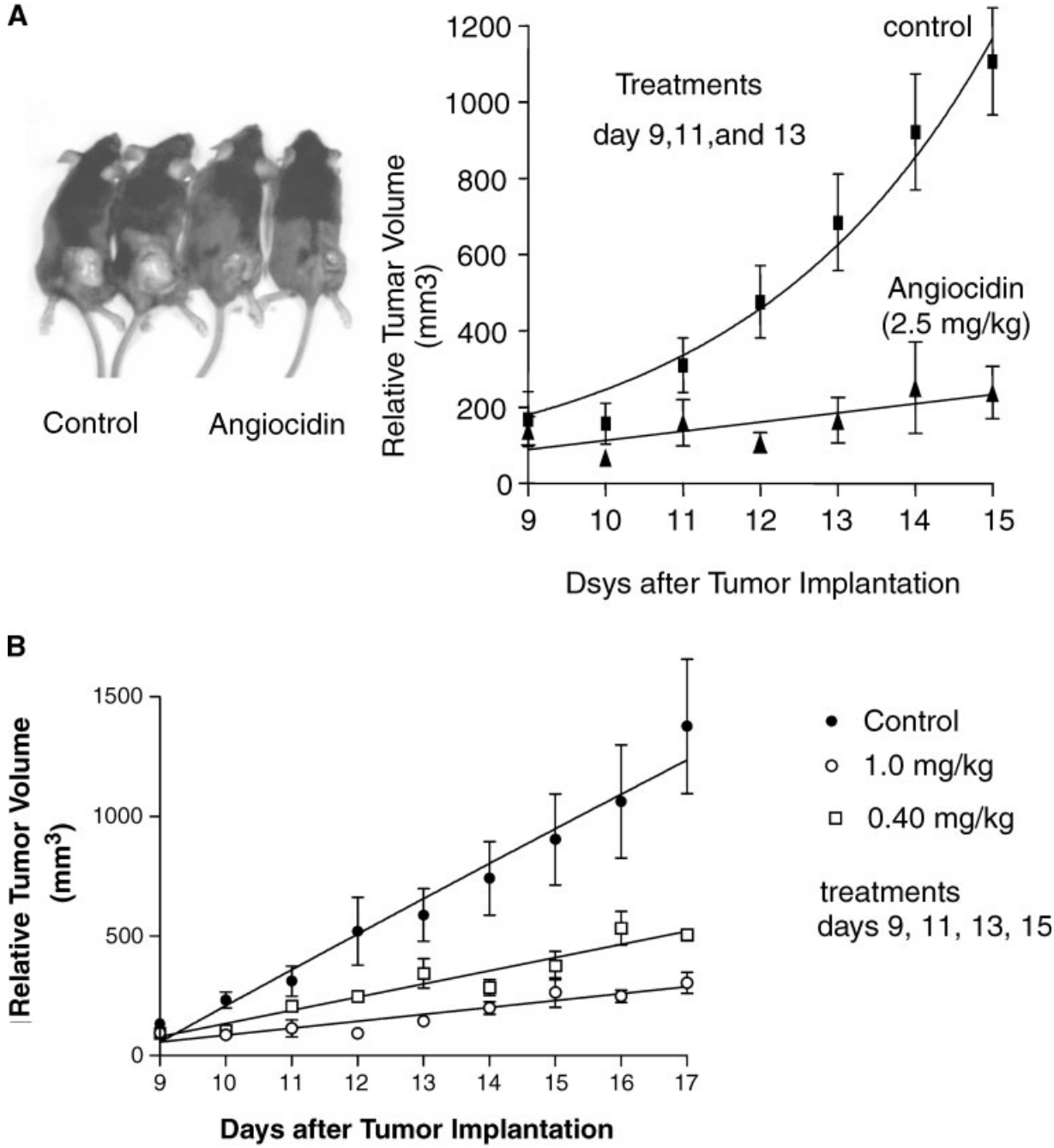

Fig. 8. Angiocidin inhibits tumor growth in vivo. **Panel A** shows a plot of the relative tumor volume measured as width^2 × length × 0.52 versus days, 9 days after tumor injection when palpable tumor appeared. Mice (five per group) were treated intravenously with 2.5 mg/kg angiocidin on days 9, 11, 13, and sacrificed on day 15. Control animals were treated with PBS. Representative animals are shown to the left of the graph with skin removed from the tumors. **Panel B** shows a similar experiment to the one described in Panel A except that the animals were treated with lower doses of intravenously injected angiocidin (1 and 0.4 mg/kg). Error bars represent the standard error of the mean.

Fig. 7. Angiocidin inhibits tumor cell invasion and angiogenesis in vitro. **Panel A**: Angiocidin inhibited invasion of Lewis Lung carcinoma cells and BAE cells. Cells were plated on collagen-coated Boyden chamber inserts in serum-free media containing 0.1% BSA and various concentrations of angiocidin. The lower chamber contained media with 2% serum. **Panel B**: HUVE cells were plated in full-media containing various concentrations of angiocidin on Matrigel-coated 96 well microtiter plates and cultured overnight. Cells were photographed under phase contrast microscopy at 100×.

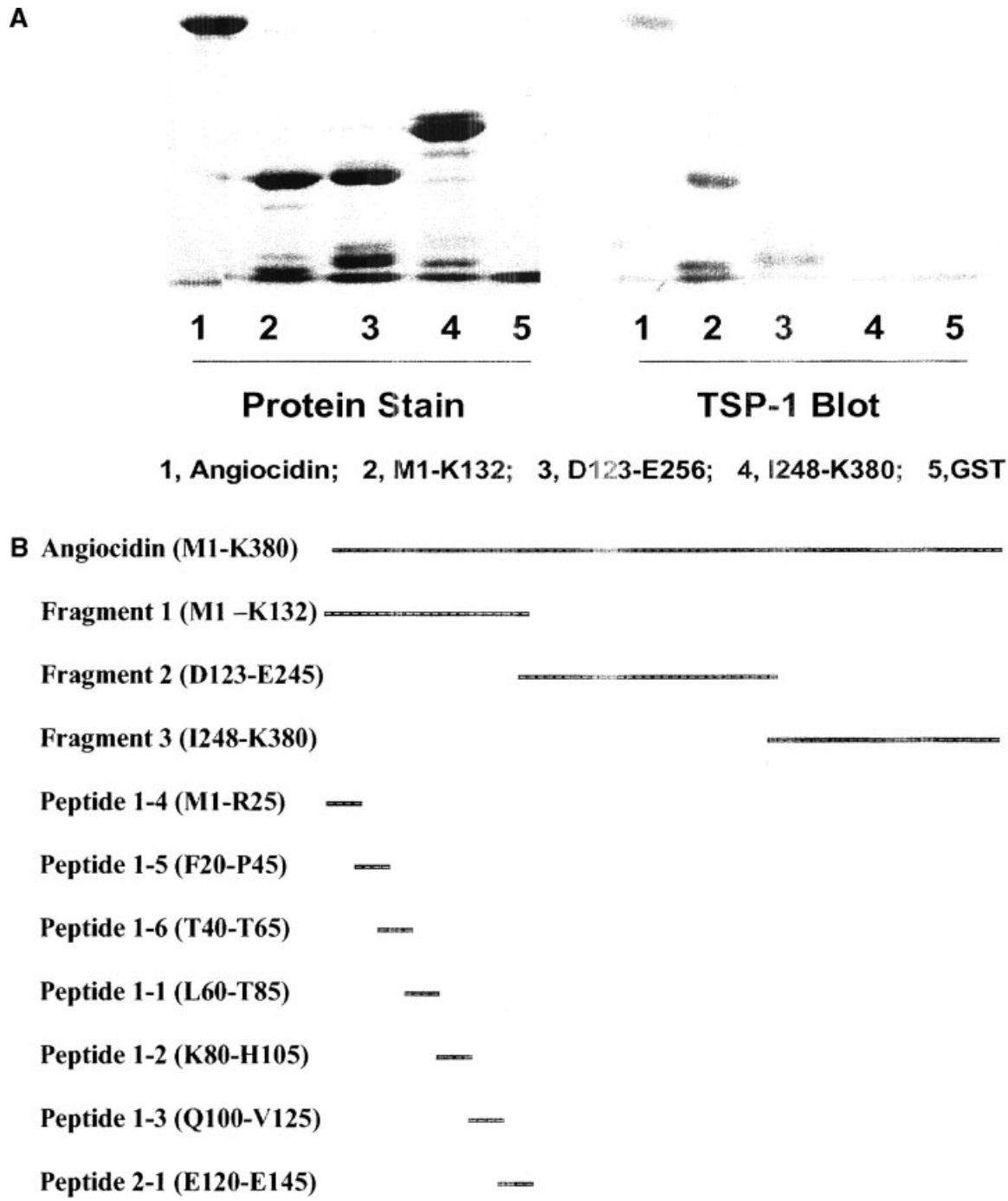

Fig. 9. Localization of the TSP-1 binding site in angiocidin. **Panel A**: TSP-1 ligand blot of overlapping GST fusion proteins corresponding to three angiocidin fragments that span the amino terminal domain (M1-K132), the middle domain (D123-E256), and carboxyl terminal domain (I248-K380). **Panel B**: Schematic representation of the overlapping peptides synthesized to localize the TSP-1 binding domain in fragment M1-K132. **Panel C**: TSP-1 binding of angiocidin and its TSP-1 deletion binding mutant M1 as measured by affinity sensor.

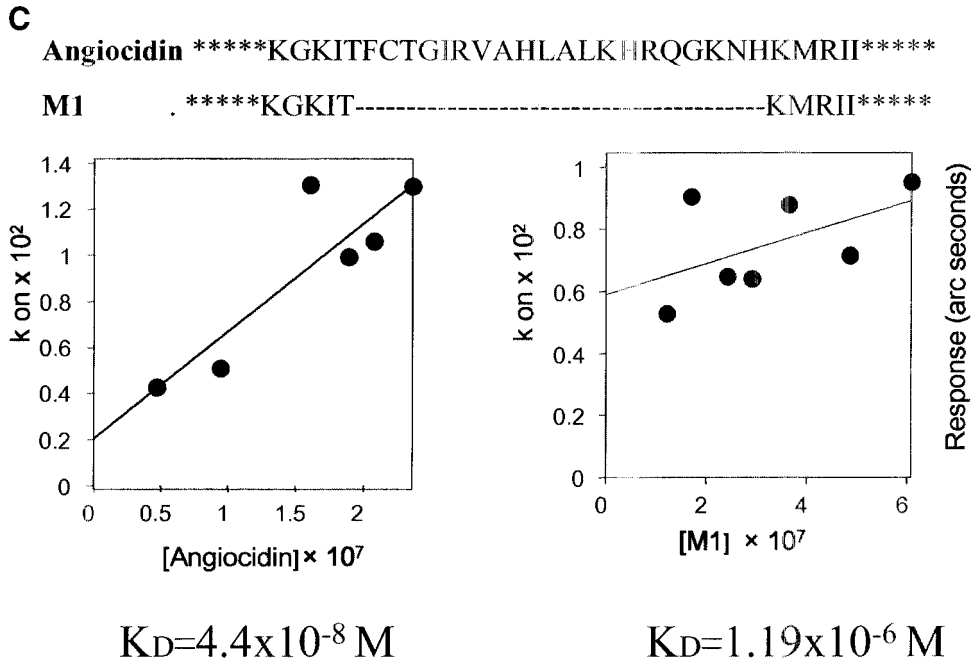

Fig. 9. (*Continued*)

shown to bind TSP-1 with a 100 fold lower affinity constant as compared to angiocidin (Fig. 9C). These results indicate that the K80-H105 sequence of angiocidin possesses significant TSP-1 binding activity.

The TSP-1 Binding Domain of Angiocidin Is Required for its Anti-Tumor Activity

We next tested the hypothesis that the TSP-1 binding contributes to the anti-tumor activity of

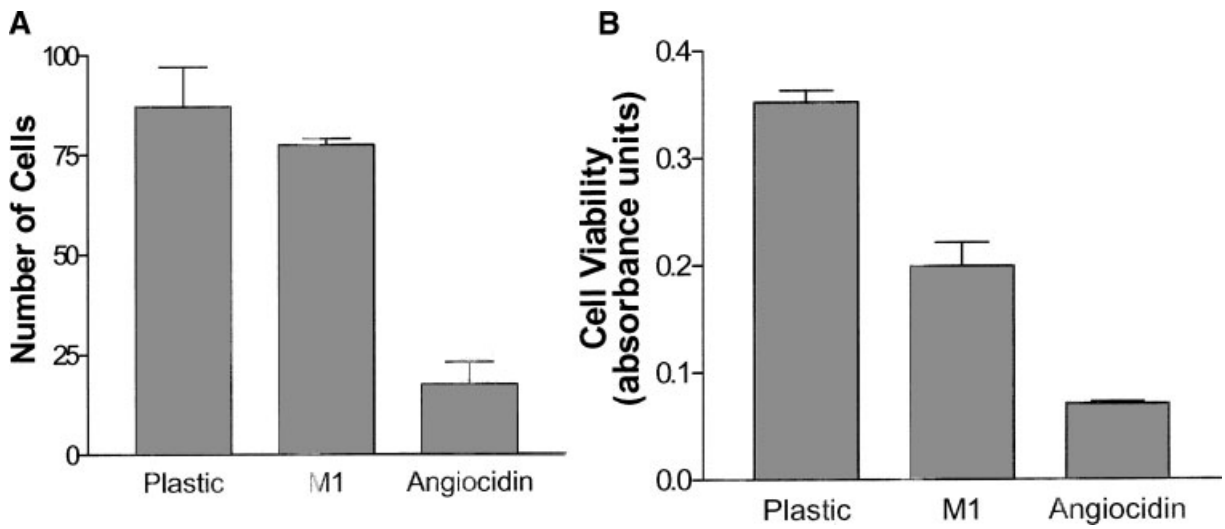

Fig. 10. The anti-tumor activity of angiocidin is dependent on its TSP-1 binding domain. **Panel A** shows that 25 μg/ml of angiocidin inhibits invasion of Lewis lung by more than 80% while M1 has little or no effect. **Panel B** shows that 100 μg/ml M1 is three times less effective in inducing HUVE cell apoptosis than angiocidin. Cell viability was measured using the Alamar Blue assay. **Panel C** shows that 3 μg of angiocidin inhibited bFGF induced angiogenesis in the chick CAM assay by more than 50% while M1 showed less than 15% inhibition as measured by radial vessel count. Angiocidin treated cams frequently showed hemorrhage under the paper disk containing angiocidin as is evident in the photomicrograph. CAMs were photographed at 2× magnification. **Panel D** shows the relative tumor volume of animals treated with 2.5 mg/kg angiocidin or M1 after 15 days as described in the legend of Figure 8. The error bars represent the standard error of the mean and each treatment group contained five animals.

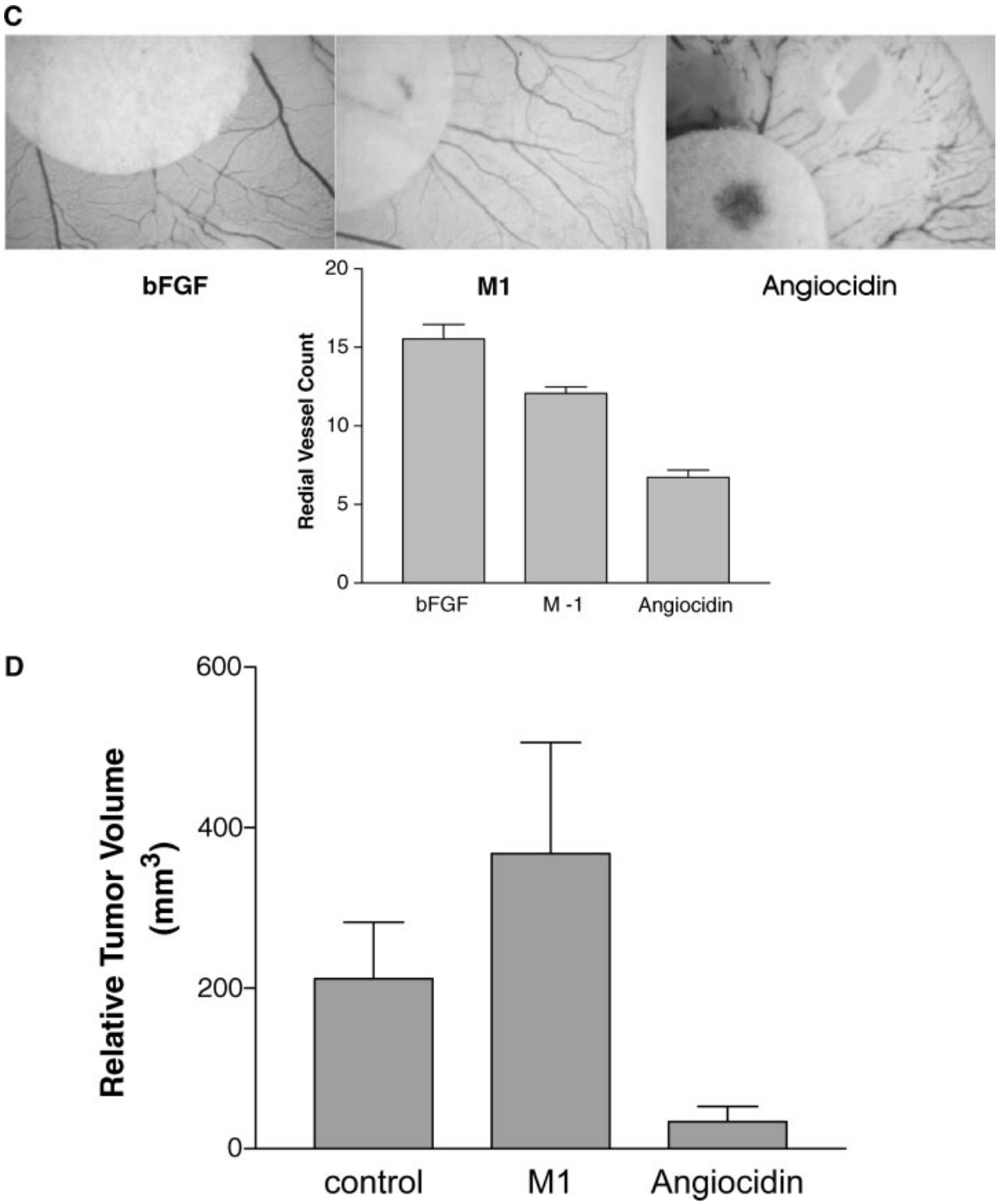

Fig. 10. (*Continued*)

angiocidin. The activity of angiocidin and its TSP-1 deficient binding mutant M1 was compared in a number of in vitro and in vivo assays (Fig. 10). We found that M1 lacked inhibitory activity in Lewis Lung tumor cell invasion of collagen (Fig. 10A). M1 was three times less active in promoting HUVE cell death (Fig. 10B) and displayed virtually no anti-angiogenic activity in the chick CAM assay (Fig. 10C). Finally, M1 had no anti-tumor activity in preventing the growth of subcutaneously implanted Lewis lung carcinoma in mice

(Fig. 10D). These data strongly support the hypothesis that the anti-tumor activity of angiocidin depends on its capacity to interact with TSP-1.

DISCUSSION

TSP-1 is a matrix protein that functions in mechanisms of tumor progression and angiogenesis. It has generally been accepted that TSP-1 inhibits tumor growth and angiogenesis based on a number of studies showing that TSP-1 over-expressing tumors grow more slowly and are less vascularized than their low expressing controls [Lawler, 2002]. However, a number of studies argue that TSP-1 may also promote angiogenesis. BenEzra et al. [1993] reported that TSP-1 greatly potentiates (fivefold) the angiogenic effect of bFGF in the rabbit cornea model. These findings are consistent with our studies demonstrating that matrix-bound TSP-1 stimulates angiogenesis in the rat aorta model [Nicosia and Tuszynski, 1994]. Moreover, Taraboletti et al. [1990] showed that TSP-1 promotes endothelial cell migration, a process which is essential during angiogenesis and that TSP-1 promotes angiogenesis in the rabbit cornea assay. These authors localized the TSP-1 angiogenesis promoting domain to the 25 kDa amino terminus. Additional evidence that TSP-1 may play a potentiating role in microvessel formation was obtained by RayChaudhury et al. [1994] who showed that virus-transformed endothelial cells incapable of forming microvessels expressed little or no TSP-1 mRNA, which was instead expressed at high level in normal microvascular endothelial cells. In addition, activated monocytes, which stimulate angiogenesis in vivo, upregulate production of TSP-1 sixfold, as compared to unstimulated monocytes which are not angiogenic [DiPietro and Polverini, 1993].

Although considerable evidence has accumulated in the literature indicating that TSP-1 is up-regulated in the highly vascularized stroma of breast cancer [Wong et al., 1992; Tuszynski and Nicosia, 1994] and other cancers such as colon and pancreatic [Qian et al., 2001; Wakiyama et al., 2001] a gap exists in our understanding of the role of this molecule in cancer-related angiogenesis and invasion. In an attempt to further provide an explanation for the inhibitory effects of TSP-1 in tumor growth, we have isolated a TSP-1 binding protein that inhibits tumor growth and angiogenesis through its capacity to interact with TSP-1, thus conferring TSP-1 anti-tumor activity. The molecule which we have named, angiocidin, is identical to the protein we isolated by CSVTCG-column chromatography more than 10 years ago [Tuszynski et al., 1993]. The full-length cDNA obtained in this study reveal that angiocidin is nearly identical to S5a and anti-secretory factor. S5a is cytoplasmic while anti-secretory factor is secreted [Nilsson et al., 1992]. However, our recent data show that angiocidin is both secreted and intracellular and likely to interact with TSP-1 as discussed below. For example, cancer patients have high circulating levels of angiocidin in the blood (data not shown) as well as high levels of TSP-1 [Tuszynski et al., 1992b].

Angiocidin is over-expressed in a number of human tumors (Fig. 3) as well as common mouse tumors such as Lewis lung carcinoma and mouse melanoma (data not shown). Previous studies from our laboratory have shown that angiocidin (previously referred to as the CSVTCG receptor) is over-expressed in breast carcinoma [Tuszynski and Nicosia, 1994], head and neck cancer [Arnoletti et al., 1994], and colon carcinoma [Wakiyama et al., 2001]. In the current study using tissue arrays, we have discovered that angiocidin is over-expressed in a variety of human tumors such as lung, prostate, bladder, pancreatic, liver, melanoma, and ovarian tumors. Low expression of angiocidin was observed in normal tissue and limited to epithelial cells and stroma.

Recombinant angiocidin bound TSP-1 with high affinity (Fig. 4). The binding constant is at least an order of magnitude more favorable than that reported for CD36 [Asch et al., 1992] and integrins [Karczewski et al., 1989] suggesting that the interaction is physiologically significant. In an attempt to establish the interaction of endogenous angiocidin with endogenous TSP-1, we were able to show co-localization of TSP-1 and angiocidin in endothelial cells (Fig. 5) and breast carcinoma (data not shown). In addition, angiocidin was co-immunoprecipitated with TSP-1. These data strongly support the in vivo association of angiocidin with TSP-1.

Exogenously added recombinant angiocidin or substratum-bound angiocidin induced apoptosis in both endothelial and tumor cells grown in tissue culture (Fig. 6). The apoptotic affect of angiocidin was dose-dependent and could

be reversed with anti-angiocidin antibody. Endothelial cells were more susceptible to the apoptotic activity of exogenously added angiocidin while tumor cells plated on collagen and fibronectin were protected (data not shown). These data suggest that the activity of angiocidin may be modulated by integrin ligation.

We also investigated the effect of exogenously added angiocidin on tumor cell and endothelial invasion as well as endothelial tube formation on Matrigel. We found that angiocidin inhibited tube formation and cell invasion in a dose-dependent manner (Fig. 7). Doses of angiocidin of 25 μg/ml that did not induce apoptosis inhibited cell invasion by more than 50% indicating that angiocidin has anti-invasive activity at low concentration.

In vivo, angiocidin inhibited tumor growth in a dose-dependent manner. At doses as low as 0.40 mg/kg administered by systemic iv injection on alternating days when tumor nodules were palpable, angiocidin inhibited tumor growth by more than 70% (Fig. 8). Higher doses were even more effective. Histology of the tumor masses that developed post treatment with 2.5 mg/kg of angiocidin showed no presence of microvessels with necrotic tumors and after treatment with lower doses of angiocidin, tumors that developed contained too few microvessels to quantitate (data not shown). We saw no toxic effects in any other organs of the mice. These data indicate that angiocidin is a potent inhibitor of tumor growth effective at doses orders of magnitude lower than anti-angiogenic agents such as endostatin [O'Reilly et al., 1997] that show efficacy with continuous infusion at doses greater than 50 mg/kg.

To investigate the role of TSP-1 in the anti-tumor activity of angiocidin, we localized the TSP-1 binding domain of angiocidin and engineered a deletion mutant of angiocidin missing a 25 amino acid sequence near the amino acid terminal domain. This mutant angiocidin termed M1 bound TSP-1 100-fold lower than the parent molecule (Fig. 9C). We then compared the anti-angiogenic and anti-tumor activity of M1 and angiocidin in a number of in vitro and in vivo assays. M1 did not inhibit cell invasion of Lewis lung carcinoma at 25 μg/ml and was three times less potent than angiocidin at inducing apoptosis of HUVE cells at concentrations of 150 μg/ml (Fig. 10A,B). Similarly, M1 at concentrations of 25 μg/ml was ineffective at inhibiting bFGF angiogenesis in the chick CAM assay (Fig. 10C). Finally, in the Lewis Lung tumor model M1 showed no inhibition of tumor growth rather showing potentiation with respect to the buffer control (Fig. 10D). We do not know why mutant M1 potentiates tumor growth. Perhaps this is due to other binding activities of angiocidin that become pronounced when TSP-1 binding is prevented. Taken together, these data provide strong evidence that the TSP-1 binding domain of angiocidin is required for its anti-angiogenic and anti-tumor activity.

In summary, our data suggest that angiocidin together with TSP-1 act to suppress tumor invasion, growth, and angiogenesis. These data may explain why TSP-1 over-expressing tumors grow more slowly and are less vascularized. Angiocidin appears to be a naturally occurring angiogenesis inhibitor which is ironically produced by tumors. Our data support the hypothesis proposed by Folkman et al. [Folkman, 1995] that tumors suppress their own growth and angiogenesis through endogenous inhibitors of angiogenesis. Angiocidin being a naturally occurring molecule and having no obvious side effects in our animal studies may have considerable potential as an anti-cancer therapeutic.

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