

Angiotensin-(1-7) Reduces Fibrosis in Orthotopic Breast Tumors

Katherine L. Cook^{1,2}, Linda J. Metheny-Barlow³, E. Ann Tallant^{1,2}, and Patricia E. Gallagher¹

Abstract

Angiotensin-(1-7) [Ang-(1-7)] is an endogenous 7-amino acid peptide hormone of the renin-angiotensin system that has antiproliferative properties. In this study, Ang-(1-7) inhibited the growth of cancer-associated fibroblasts (CAF) and reduced fibrosis in the tumor microenvironment. A marked decrease in tumor volume and weight was observed in orthotopic human breast tumors positive for the estrogen receptor (BT-474 or ZR-75-1) and HER2 (BT-474) following Ang-(1-7) administration to athymic mice. Ang-(1-7) concomitantly reduced interstitial fibrosis in association with a significant decrease in collagen I deposition, along with a similar reduction in perivascular fibrosis. In CAFs isolated from orthotopic breast tumors, the heptapeptide markedly attenuated *in vitro* growth as well as reduced fibronectin, transforming growth factor- β (TGF- β), and extracellular signal-regulated kinase 1/2 kinase activity. An associated increase in the mitogen-activated protein kinase (MAPK) phosphatase DUSP1 following treatment with Ang-(1-7) suggested a potential mechanism by which the heptapeptide reduced MAPK signaling. Consistent with these *in vitro* observations, immunohistochemical analysis of Ang-(1-7)-treated orthotopic breast tumors revealed reduced TGF- β and increased DUSP1. Together, our findings indicate that Ang-(1-7) targets the tumor microenvironment to inhibit CAF growth and tumor fibrosis. *Cancer Res*; 70(21): 8319–28. ©2010 AACR.

Introduction

Cross talk between cancer cells and surrounding tissue is essential for the development and progression of tumors. The interaction between cancerous cells and the adjacent microenvironment transforms the stroma into an abnormal phenotype, altering normal function (1, 2). The altered stromal microenvironment affects tissue architecture, cellular morphology, and extracellular matrix (ECM)-cell interactions that directly contribute to formation of the neoplasia (1). Solid tumors, in particular breast tumors, are characterized by pathologic desmoplasia, resulting in increased fibrosis and ECM deposition (1, 2). About 80% of reactive stroma associated with breast carcinoma is composed of activated myofibroblasts (3), which secrete ECM proteins, resulting in desmoplasia and breast tumor progression (4).

Activated myofibroblasts play a vital role in tumor initiation, growth, and metastases. Tumor stroma myofibroblasts or “cancer-associated fibroblasts” (CAF) are characterized by their expression profile and are distinguished from nor-

mal fibroblasts by the expression of α -smooth muscle actin (α -SMA) and vimentin (5). Moreover, the presence of α -SMA-positive myofibroblasts around noninvasive epithelium in breast ductal carcinoma *in situ* is strongly correlated with the onset of tumor invasion and poor prognosis (5, 6). Inflammation and cytokine secretion by cancer cells result in the recruitment of CAF to the tumor site. Activated myofibroblasts are transformed by cytokines such as transforming growth factor- β (TGF- β) and secrete tumor-promoting growth factors including hepatocyte growth factor (HGF), platelet-derived growth factor, vascular endothelial growth factor (VEGF), basic fibroblast growth factor, TGF- α , and TGF- β (5), which leads to the production of ECM components including collagens, tenascin-C, and fibronectin (7).

CAFs play an important role in the initiation and progression of cancer; however, cancer therapies that target the tumor stroma are limited (5, 8). In this study, we assessed whether angiotensin-(1-7) [Ang-(1-7)], a 7-amino acid peptide hormone of the renin-angiotensin system with vasodilatory, antiproliferative, and antifibrotic properties (9), alters the tumor microenvironment to reduce tumor growth and fibrosis. Ang-(1-7) exerts its biological activity through a unique G protein-coupled receptor, *mas* (9, 10). The heptapeptide decreased mitogen-stimulated growth of vascular smooth muscle cells (VSMC) *in vitro* (11) and attenuated neointima formation in a balloon catheter injury model of the rat carotid artery, with no effect on the underlying media layer, indicating that the antigrowth properties of the heptapeptide are limited to proliferating cells (12). In a retrospective study of hypertensive patients treated with angiotensin-converting enzyme (ACE) inhibitors that increase Ang-(1-7), ACE inhibitor

Authors' Affiliations: ¹Hypertension and Vascular Research Center, ²Molecular Medicine and Translational Science Program, and ³Department of Radiation Oncology, Wake Forest University School of Medicine, Winston-Salem, North Carolina

Corresponding Author: Patricia E. Gallagher, Hypertension and Vascular Research Center, Wake Forest University School of Medicine, Medical Center Boulevard, Winston Salem, NC 27157. Phone: 336-716-4455; Fax: 336-716-2456; E-mail: pgallagh@wfbumc.edu.

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treatment reduced the risk of cancer, particularly lung and breast cancer (13). More importantly, we showed that Ang-(1-7) significantly decreased the proliferation of human lung cancer cells *in vitro* by reducing mitogen-activated protein kinase (MAPK) activity (14). Ang-(1-7) infusion decreased human A549 lung adenocarcinoma xenograft growth with a corresponding reduction in cyclooxygenase-2 (15). Lung tumors from mice injected with the heptapeptide had reduced vessel density with an associated decrease in VEGF, indicating that Ang-(1-7) inhibited tumor angiogenesis (16). These data suggested that Ang-(1-7) also regulates the tumor microenvironment to inhibit cancer growth.

Fibrosis correlates with the progression and invasion of breast cancer. The increased ECM deposition and secretion of growth factors by myofibroblasts directly contribute to breast tumor growth by stimulating tumor cell proliferation, increasing angiogenesis, and promoting invasion (5). The purpose of this study was to determine whether Ang-(1-7) could serve as an antifibrotic agent that targets the tumor microenvironment to reduce breast cancer tumor growth and fibrosis.

Materials and Methods

Materials

The following materials were purchased from the companies in the parentheses: Ang-(1-7) and [D-Ala⁷]-Ang-(1-7) (Bachem); [D-Pro⁷]-Ang-(1-7) (GenScript Corp.); penicillin, RPMI 1640, DMEM/F12, streptomycin, fetal bovine serum (FBS), and hypoxanthine-aminopterin-thymidine supplement (Life Technologies, Invitrogen); TGF- β (Calbiochem); Matrigel (BD Biosciences); picric acid (Sigma-Aldrich); saturated picric acid (LabChem, Inc.); and collagenase, trypsin, and soybean trypsin inhibitor (Worthington Biochemical). Antibodies were obtained from the following sources: collagen I and TGF- β for immunohistochemistry (Abcam); phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) and TGF- β for Western blot hybridization (Cell Signaling Technology); MKP-1 for Western blot hybridization (Upstate Biotechnology) and for immunohistochemistry (Santa Cruz Biotechnology); fibronectin, vimentin, α -SMA, and β -actin (Sigma-Aldrich); Cy2 FITC-coupled donkey anti-rabbit and Cy3 FITC-coupled donkey anti-mouse (The Jackson Laboratory); and polyclonal and horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare).

Cell culture

ZR-75-1 breast ductal carcinoma cells [American Type Culture Collection (ATCC) CRL-1500], derived from a 63-year-old Caucasian female, and BT-474 breast carcinoma cells (ATCC HTB-20), derived from a 60-year-old Caucasian female, were grown in RPMI 1640 containing 10% FBS, 100 μ g/mL penicillin, 100 units/mL streptomycin, and 10 nmol/L HEPES. Isolated tumoral fibroblasts were grown in DMEM/F12 medium containing 10% FBS, 100 μ g/mL penicillin, 100 units/mL streptomycin, and 10 nmol/L HEPES. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂/95% room air.

Orthotopic model of human breast cancer

Female athymic mice (15–20 g, 4–6 weeks of age; Charles River Laboratories) were housed in cages with high-efficiency particle arrest–filtered air (12-hour light dark cycle) and provided *ad libitum* access to food and autoclaved water. All procedures complied with the policies of the Wake Forest University Animal Care and Use Committee. Female athymic mice were ovariectomized and supplemented with 0.18 mg 17 β -estradiol 90-day time release pellets (Innovative America) to prevent cycling of hormones (17, 18). Actively growing ZR-75-1 cells (2×10^6) or BT-474 cells (5×10^6) at 75% confluence were suspended in 50% PBS/50% Matrigel and injected into the inguinal mammary fat pad (19, 20). Tumor size was measured every other day using a caliper, and tumor volume was calculated using the formula $V = [(length \times width^2)/2]$. When tumors reached a volume of 100 mm³ (ZR-75-1) or 200 mm³ (BT-474), the mice were implanted with osmotic minipumps for s.c. release of saline or 24 μ g/kg/h Ang-(1-7). After 18 days of treatment, the animals were sacrificed and tumors were excised.

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded tumors were cut into 5- μ m-thick sections and stained with H&E to determine morphology. Interstitial and perivascular fibrosis was measured by picrosirius red histochemical staining (21). Immunostaining was performed with an antibody to collagen I (1:100) using the streptavidin-biotin method (22). Immunostaining for TGF- β (1:100), DUSP1 (1:200), and vimentin (1:100) was performed using FITC-coupled Cy2 or Cy3 as secondary antibodies and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Stained sections were visualized with a Leica DM 4000 microscope ($A_{max} = 492$ nm Cy2, $A_{max} = 550$ nm Cy3 for fluorescence) and photographed with a QImaging Retiga 1300R camera. A computer-assisted counting technique with a pixel counter to select stained fibers was used to quantify picrosirius red and collagen I staining. Interstitial fibrosis was expressed as a percentage of reactive fibers/field (four fields/tumor section/mouse), whereas perivascular fibrosis was expressed as a percentage of reactive fibers/blood vessel (four vessels/tumor section/mouse).

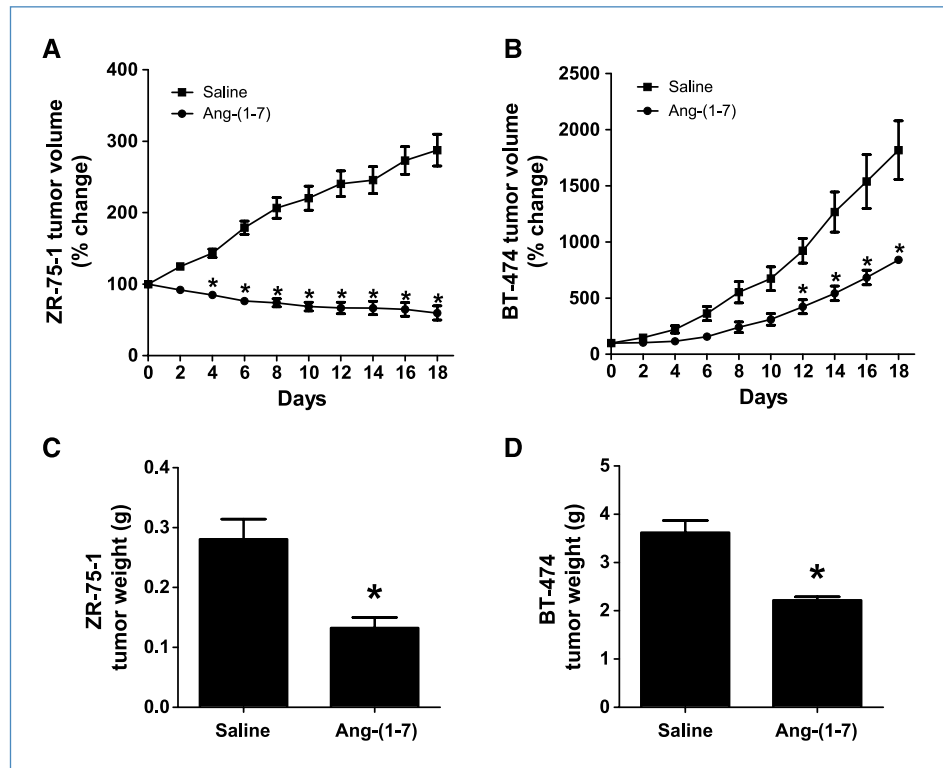
Tumor fibroblast isolation

Orthotopic ZR-75-1 breast tumors (200 mm³) were excised from mammary fat pads. Minced tumors were digested overnight at 4°C with trypsin (50 μ g/mL), and soybean trypsin inhibitor (100 μ g/mL) was added to stop the reaction. The minced tissue, harvested by centrifugation, was further digested with collagenase (85 units/mL) at 37°C for 30 minutes. Undigested tumor tissue was removed with a cell strainer, and tumor fibroblasts were isolated by differential plating as previously described (23).

Quantification of cell number

Isolated tumor fibroblasts (1×10^4 cells/mL) in DMEM/F12 containing 0.5% FBS and 10 ng/mL TGF- β were treated with PBS or 100 nmol/L Ang-(1-7) in PBS, added daily due to degradation of the heptapeptide. Cell number was determined using a hemocytometer.

Figure 1. Effect of Ang-(1-7) on orthotopic human breast cancer growth. The volumes of human ZR-75-1 (A) or BT-474 (B) orthotopic breast tumors from mice treated with saline or Ang-(1-7) were measured using a caliper, calculated as $V = 1/2(L \times W^2)$, and presented as the percent change compared with initial tumor volume. ZR-75-1 (C) or BT-474 (D) tumor weight from mice infused with saline or Ang-(1-7) was determined at time of sacrifice ($n = 5-6$). *, $P < 0.05$.



Immunofluorescence

Fibroblasts were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated overnight at 4°C with antibodies to fibronectin, vimentin, α -SMA, or collagen I (1:100). Following a 30-minute incubation with Cy2- or Cy3-labeled antibodies (1:100), the cells were counterstained with DAPI.

Western blot hybridization

Treated cell monolayers were solubilized in lysis buffer (14), and protein was measured by a modification of the Lowry method (24). Proteins were separated by PAGE and transferred to hydrophobic polyvinylidene difluoride membrane. Nonspecific binding was blocked by incubation with Blotto (TBS with 5% powdered milk and 0.1% Tween 20). Membranes were incubated overnight at 4°C with primary antibodies specific to DUSP1 (MKP-1, 1:1,000), fibronectin (1:5,000), and pERK1/2 or TGF- β (1:1,000), followed by a 1-hour incubation with polyclonal HRP-conjugated secondary antibodies (1:2,000) at room temperature. Immuno-reactive products were visualized by chemiluminescence (SuperSignal Femto or Pico West, Pierce Biotechnology) and quantified by densitometry using MCID digital densitometry software. Protein loading was visualized by incubation of stripped membranes with a monoclonal antibody to β -actin (1:2,000).

Statistics

All data are presented as the mean \pm SE. Statistical differences were evaluated by Student's *t* test or one-way ANOVA

followed by Dunnett's post hoc test. The criterion for statistical significance was set at $P < 0.05$.

Results

Inhibition of orthotopic human breast tumors by Ang-(1-7)

Athymic mice bearing human ZR-75-1 or BT-474 orthotopic breast tumors were administered saline or 24 μ g/kg/h Ang-(1-7) via osmotic minipump. The ZR-75-1 tumor volume in the two treatment groups was not statistically different at the beginning of treatment [117.3 ± 4.3 mm³ in the saline-treated group when compared with 123.8 ± 7.6 mm³ in the Ang-(1-7)-treated group]. Tumor volume of saline-medicated mice continued to increase, whereas the growth of tumors in Ang-(1-7)-treated mice was significantly inhibited beginning at day 4 of treatment [day 18 tumor volumes: $287.4 \pm 22.2\%$ change in the saline-treated mice compared with $59.7 \pm 9.8\%$ change in the Ang-(1-7)-treated animals when compared with original tumor volume; $P < 0.001$; Fig. 1A]. By the end of the study, the volume of ZR-75-1 tumors from Ang-(1-7)-medicated mice was reduced by 25% compared with the initial tumor volume, resulting in a %T/C [(average relative tumor volume of the treated group/average relative tumor volume of the control group) \times 100] of 22.3%. Due to the diminished size of the tumors from mice administered Ang-(1-7), the study was terminated at 18 days to provide sufficient tumor tissue for mechanistic studies.

The tumor volume of the two treatment groups of athymic mice injected with human BT-474 breast cancer cells was not statistically different at the beginning of treatment

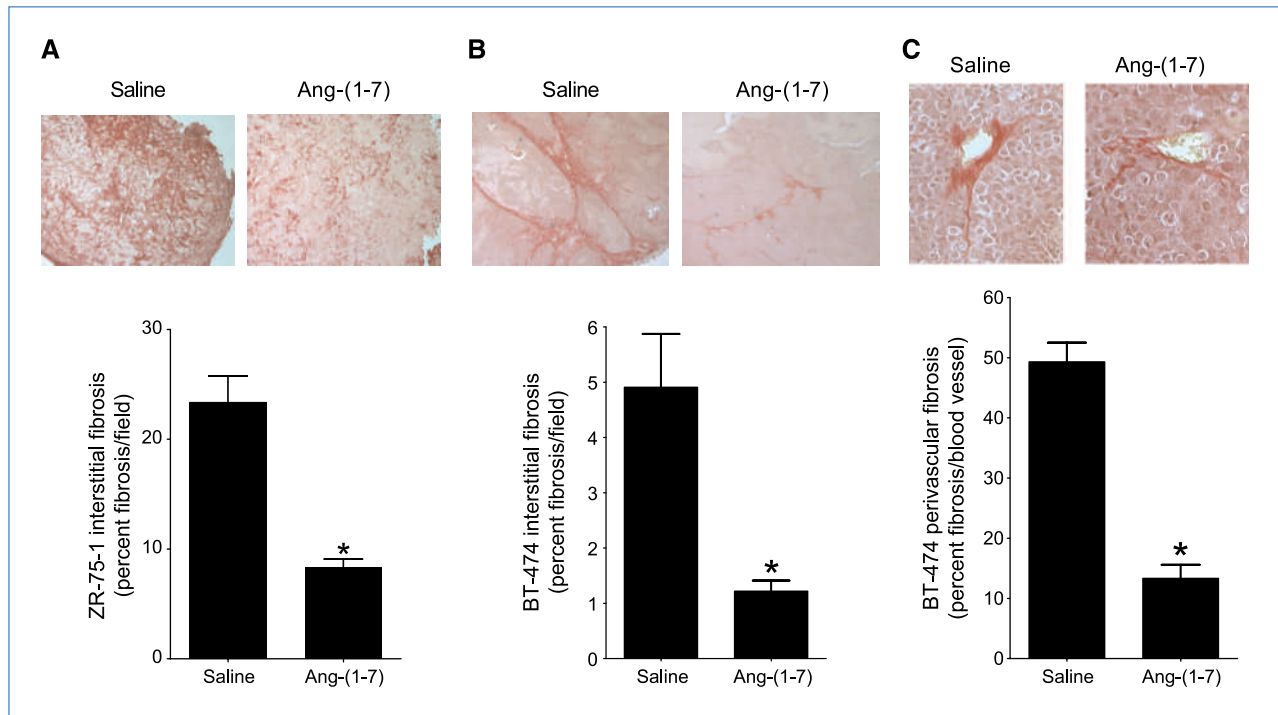


Figure 2. Inhibition of breast tumor fibrosis by Ang-(1-7). Representative pictures of picrosirius red–stained sections of ZR-75-1 (A) and BT-474 (B) tumors from mice treated with saline or Ang-(1-7) are shown at $\times 200$ magnification. Interstitial fibrosis was calculated as the percent fibrosis per field in sections of ZR-75-1 or BT-474 tumors. C, representative pictures of picrosirius red–stained blood vessels from BT-474 tumors at $\times 200$ magnification. Perivascular fibrosis was calculated as percent fibrosis per vessel ($n = 5-6$). *, $P < 0.05$.

[$327.0 \pm 72.5 \text{ mm}^3$ in the saline-treated group compared with $197.7 \pm 15.9 \text{ mm}^3$ in the Ang-(1-7)–administered group]. The volume of the tumors in saline-treated mice continued to increase, whereas the growth of tumors from the heptapeptide-medicated animals was inhibited significantly beginning at day 12 [final tumor volumes: $1,818 \pm 260.2\%$ change in the saline-treated animals compared with $841.2 \pm 24.9\%$ change in the Ang-(1-7)–treated animals when compared with initial tumor volumes; $P < 0.001$; Fig. 1B]. Ang-(1-7) administration of BT-474 orthotopic human breast tumors resulted in a %T/C of 29.9%. The study was terminated at 18 days for health concerns due to the large tumor burden of the saline-treated mice.

No gross pathologic side effects were observed with heptapeptide administration; there was no change in motor function, body weight, and food and water intake. No metastases were observed in mice with ZR-75-1 or BT-474 tumors due to the short time of the study. On completion of the study, the mice were euthanized and tumors were excised and weighed. The ZR-75-1 tumors from mice treated with Ang-(1-7) weighed $\sim 50\%$ less than the tumors from the saline control animals ($0.29 \pm 0.03 \text{ g}$ versus $0.14 \pm 0.02 \text{ g}$; Fig. 1C). Similarly, the BT-474 tumors from mice that were medicated with Ang-(1-7) weighed $\sim 40\%$ less than the tumors from the mice treated with saline ($3.62 \pm 0.25 \text{ g}$ versus $2.22 \pm 0.07 \text{ g}$; Fig. 1D).

Inhibition of tumor fibrosis by Ang-(1-7)

Interstitial tumoral fibrosis was quantified in orthotopic breast tumor sections stained with picrosirius red, a non-

specific collagen stain. ZR-75-1 and BT-474 tumors from saline-treated mice displayed heavy deposits of collagen, which were reduced by Ang-(1-7) administration (Fig. 2A and B). The relative amount of picrosirius red staining was quantified, and the amount of collagen within the tumors was expressed as percent fibrosis per field. Treatment with the heptapeptide reduced interstitial fibrosis by 64% in the ZR-75-1 tumors ($23.3 \pm 2.4\%$ versus $8.3 \pm 0.8\%$ fibrosis per field; Fig. 2A) and by 75% in the BT-474 tumors ($4.9 \pm 1.0\%$ versus $1.2 \pm 0.2\%$ fibrosis per field; Fig. 2B), indicating that Ang-(1-7) reduces tumor fibrosis in orthotopic breast tumors. ZR-75-1 tumors from saline-treated mice had 3-fold more interstitial fibrosis when compared with BT-474 tumors from saline-treated mice, showing a differential deposition of collagen between the two types of breast tumors. The amount of picrosirius red staining surrounding blood vessels in BT-474 tumors was also quantified to measure perivascular fibrosis. It was not possible to quantify perivascular fibrosis in ZR-75-1 tumors due to pervasive interstitial fibrosis throughout the tumor. Heavy deposits of collagen were visualized around blood vessels in BT-474 tumors from saline-treated animals, whereas tumor sections from Ang-(1-7)–medicated animals had reduced perivascular fibrosis. The heptapeptide decreased collagen deposition around the blood vessels by 73% ($49.3 \pm 3.2\%$ versus $13.4 \pm 2.2\%$ fibrosis per blood vessel; Fig. 2C), suggesting that Ang-(1-7) reduces both interstitial and perivascular fibrosis in breast tumors.

Interstitial fibrosis in orthotopic breast tumors was further characterized by the immunoreactivity of collagen I, one of the main isoforms of collagen found in fibrotic breast tissue. Representative pictures of collagen I immunoreactivity in ZR-75-1 and BT-474 tumor sections are shown in Fig. 3A and B, respectively. Ang-(1-7) reduced collagen I deposition by 80% in ZR-75-1 orthotopic breast tumors [$16.2 \pm 1.7\%$ per field in the tumors of saline-treated mice versus $3.3 \pm 0.9\%$ per field in tumors from Ang-(1-7)-treated mice], whereas a 78% decrease in collagen I deposition was observed in the BT-474 tumors [$3.3 \pm 0.7\%$ per field in the tumors of saline-treated animals when compared with $0.7 \pm 0.1\%$ per field in tumors from Ang-(1-7)-medicated animals].

Inhibition of tumor-associated fibroblast growth by Ang-(1-7)

Fibroblasts were isolated from ZR-75-1 tumors to identify the molecular mechanism for the Ang-(1-7)-mediated reduction in tumor fibrosis. Tumor-associated fibroblasts, isolated by differential plating, were characterized as myofibroblasts by positive immunoreactivity to fibronectin, vimentin, collagen I, and α -SMA (Fig. 4A). The percent of

cells that showed positive immunoreactivity for fibronectin, vimentin, and α -SMA was determined in sequentially passaged tumoral fibroblasts to determine whether the cells maintain their phenotype as activated myofibroblasts with time in culture. As shown in Fig. 4B, fibroblasts isolated from orthotopic tumors retained the activated myofibroblast phenotype until passage 5; therefore, only cells from passages 2 to 4 were used for *in vitro* experiments.

Isolated tumoral fibroblasts were treated with PBS or 100 nmol/L Ang-(1-7) daily for 10 days, and the cells were counted using a hemocytometer, as a measure of cell proliferation. Ang-(1-7) significantly reduced the growth of cultured myofibroblasts isolated from orthotopic breast tumors at days 4, 7, and 10, with a 33% reduction in cell growth at day 10 [$10,700 \pm 400$ PBS-treated myofibroblasts versus $7,000 \pm 200$ Ang-(1-7)-treated myofibroblasts; Fig. 5A].

Ang-(1-7) reduces MAPK activity by upregulation of a MAPK phosphatase

pERK1/2 is a potent mitogenic signaling protein implicated in cell survival, growth, and proliferation. pERK1/2 was measured by Western blot hybridization in protein homogenates

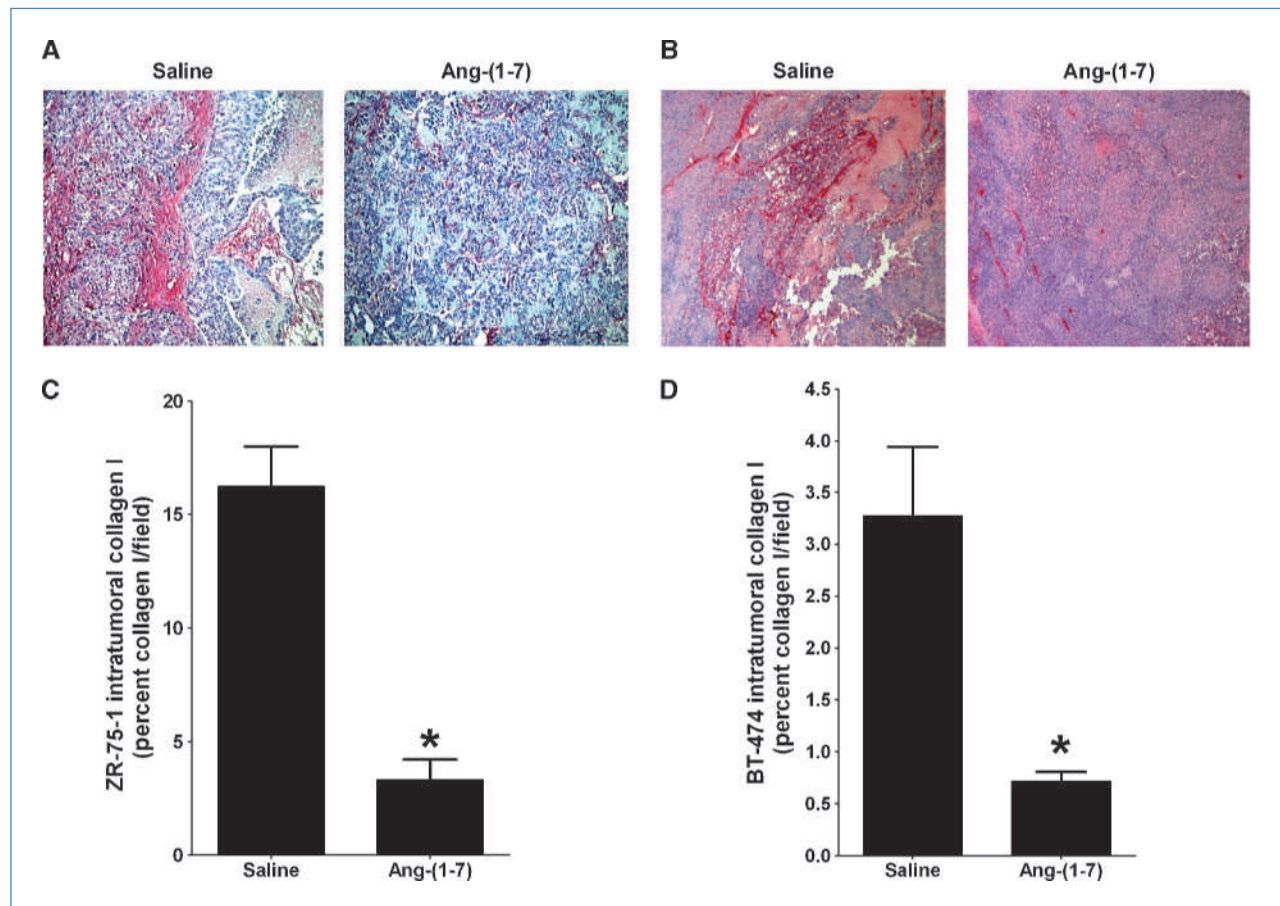


Figure 3. Ang-(1-7) reduction in collagen I deposition in breast tumors. ZR-75-1 (A) or BT-474 (B) tumors from mice treated with saline or Ang-(1-7) were incubated with an antibody to collagen I, and representative pictures are shown at $\times 200$ magnification. Collagen deposition was quantified as the percent collagen I per field in ZR-75-1 (C) and BT-474 (D) tumors ($n = 5-6$). *, $P < 0.05$.

from myofibroblasts stimulated with 10 ng/mL TGF- β and treated with PBS or 100 nmol/L Ang-(1-7) to determine if the heptapeptide regulates phosphorylated MAPKs. Ang-(1-7) decreased pERK1 by 47% and pERK2 by 63% [1.13 ± 0.23 relative density units (RDU) in PBS-treated cells versus 0.60 ± 0.06 RDU in Ang-(1-7)-treated cells for pERK1, and 2.27 ± 0.56 RDU in PBS-treated cells versus 0.84 ± 0.08 RDU in Ang-(1-7)-treated cells for pERK2; Fig. 5B], indicating that the Ang-(1-7)-mediated antiproliferative effect may be due to a reduction in pERK1/2.

MAPKs are phosphorylated and activated by MAPK kinases and dephosphorylated and inactivated by MAPK phosphatases. DUSP1 (dual specificity phosphatase 1), a MAPK phosphatase that dephosphorylates and inactivates ERK1/2, was upregulated 2.52 ± 0.29 -fold by Ang-(1-7) in tumor-associated fibroblasts, suggesting that the heptapeptide may reduce pERK1/2 by upregulation of the MAPK phosphatase DUSP1 (Fig. 5C). Pretreatment with the Ang-(1-7) receptor [AT_{(1-7)R}] antagonist D-[Ala⁷]-Ang-(1-7) or D-[Pro⁷]-Ang-(1-7) (100 nmol/L) completely blocked

the Ang-(1-7)-mediated increase in DUSP1 (Fig. 5D), whereas the antagonists alone had no effect, indicating that Ang-(1-7) activated an AT₍₁₋₇₎ receptor to increase DUSP1 in tumor-associated fibroblasts.

Ang-(1-7) reduces TGF- β 1 and fibronectin in tumoral fibroblasts

TGF- β 1 is a potent stimulator of fibroblast activation that transforms fibroblasts to secreting myofibroblasts. TGF- β 1 was quantified by Western blot hybridization in protein homogenates from myofibroblasts isolated from orthotopic breast tumors and treated with PBS or 100 nm Ang-(1-7) for 24 hours. Ang-(1-7) reduced TGF- β 1 by $45.4 \pm 11.7\%$ in myofibroblasts compared with controls (Fig. 6A). Because TGF- β 1 stimulates myofibroblasts to synthesize and deposit ECM proteins, myofibroblasts were stimulated with 10 ng/mL TGF- β 1 and treated with either PBS or 100 nmol/L Ang-(1-7) for 24 hours to determine if the heptapeptide regulated fibronectin synthesis. Ang-(1-7) administration decreased fibronectin by $37.5 \pm 12.0\%$ compared with the control (Fig. 6B), suggesting that Ang-(1-7) reduces fibronectin synthesis to attenuate ECM protein deposition, fibrosis, and fibronectin signaling.

Sections from ZR-75-1 tumors in mice treated with saline or Ang-(1-7) were incubated with antibodies to TGF- β 1 (Fig. 6C) or DUSP1 (Fig. 6D) and vimentin. TGF- β 1 immunoreactivity was reduced and DUSP1 immunoreactivity was increased in perivascular fibroblasts identified by positive immunoreactivity to the vimentin antibody and by vascular morphology in mice treated with Ang-(1-7) compared with saline, in agreement with studies in isolated tumor-associated fibroblasts.

Discussion

The link between fibrosis and breast cancer is well established. Collagen I deposition in the breast leads to increased mammographic density, which correlates with breast cancer risk (25). Increased ECM remodeling and stiffening by collagen and fibronectin enhances tumor cell survival and proliferation (26). Moreover, the secretion of growth factors, cytokines, and proangiogenic peptides by myofibroblasts promotes breast tumor growth (5). The present study is the first to show that Ang-(1-7) reduces the growth of human breast orthotopic tumors with a corresponding decrease in tumoral fibrosis. The reduction in interstitial and perivascular fibrosis was associated with a decrease in collagen I deposition. The heptapeptide reduced the serum-stimulated proliferation of isolated tumoral fibroblasts, suggesting that Ang-(1-7) inhibits myofibroblast growth to reduce tumor fibrosis. The reduction in fibroblast proliferation by Ang-(1-7) was associated with increased DUSP1 and a corresponding decrease in pERK1/2. The upregulation of DUSP1 was blocked by the AT₍₁₋₇₎ receptor antagonist, indicating that DUSP1 induction by Ang-(1-7) was a receptor-mediated process. Ang-(1-7) also reduced production of active TGF- β and synthesis of fibronectin in the isolated tumoral fibroblasts. This ability of Ang-(1-7) to prevent production of TGF- β 1

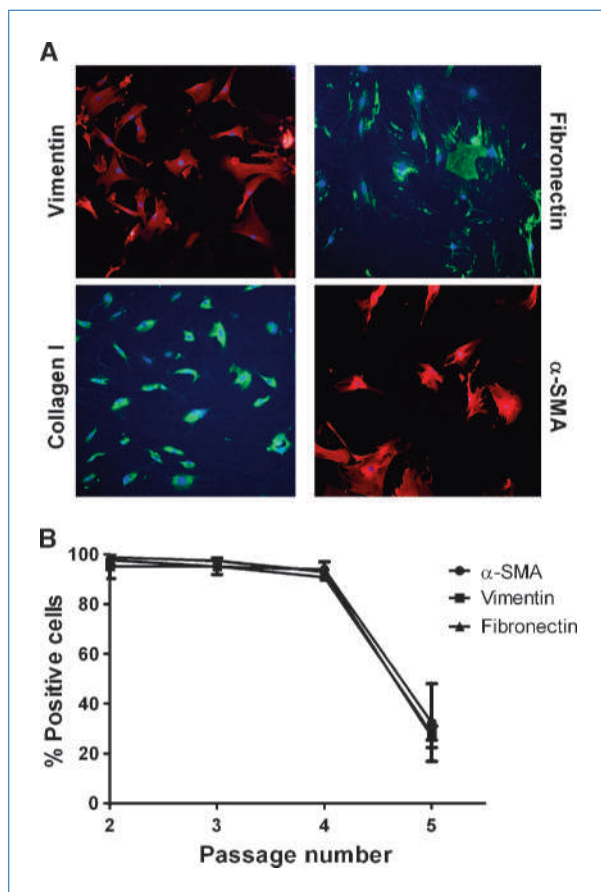
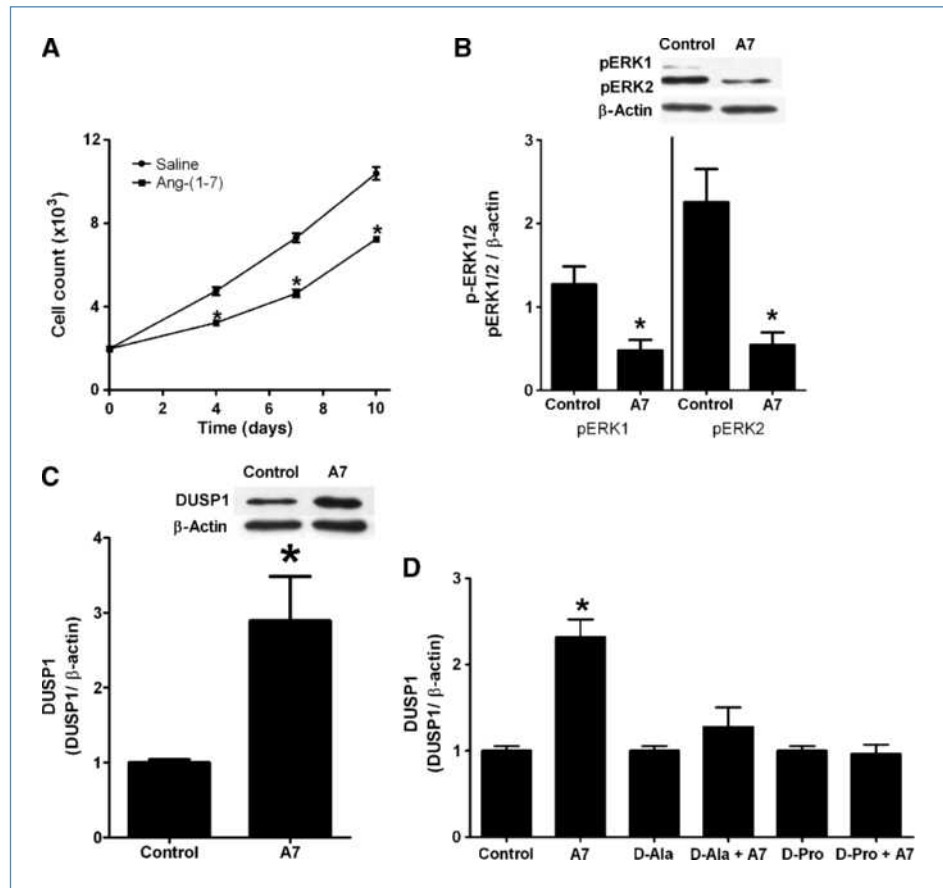


Figure 4. Characterization of isolated tumoral fibroblasts. A, isolated tumoral fibroblasts were incubated with antibodies to vimentin, fibronectin, collagen I, and α -SMA. B, the percent of cells that showed positive immunoreactivity for fibronectin, vimentin, and α -SMA was determined in sequentially passaged tumoral fibroblasts ($n = 2-3$).

Figure 5. Ang-(1-7) inhibition of isolated tumor myofibroblast growth. A, tumoral fibroblasts were treated with PBS or 100 nmol/L Ang-(1-7) and 2% FBS, and cell number was determined on days 4, 7, and 10 using a hemocytometer. B, tumoral fibroblasts were serum starved overnight, pretreated with PBS (control) or 100 nmol/L Ang-(1-7) for 4 h, and stimulated with 10 ng/mL TGF- β 1 for 2 h. pERK1/2 was assessed by Western blot hybridization and is presented as a function of β -actin. C, tumoral fibroblasts were serum starved overnight and treated with PBS (control) or 100 nmol/L Ang-(1-7) for 4 h. DUSP1 was assessed by Western blot hybridization and presented as a function of β -actin. D, tumoral fibroblasts were incubated for 4 h with PBS (Control), 100 nmol/L Ang-(1-7) (A7), 1 μ mol/L D-[Ala⁷]-Ang-(1-7) (D-Ala), 100 nmol/L Ang-(1-7) and 1 μ mol/L D-[Ala⁷]-Ang-(1-7) (D-Ala + A7), 1 μ mol/L D-[Pro⁷]-Ang-(1-7) (D-Pro), and 100 nmol/L Ang-(1-7) and 1 μ mol/L D-[Pro⁷]-Ang-(1-7) (D-Pro + A7). DUSP1 was assessed by Western blot hybridization ($n = 3-4$ of cells from different passage numbers isolated from three different tumors). *, $P < 0.05$.



as well as decrease TGF- β 1-stimulated ERK1/2 activation and fibronectin synthesis indicates that the heptapeptide is an antagonist of TGF- β 1-mediated fibrosis in breast cancer. Of note, Ang-(1-7) also reduced the proliferation of breast tumor cell *in vitro*; the molecular mechanisms involved in the inhibition of breast cancer cell proliferation are detailed in a separate article.⁴

Collagen I deposition was significantly decreased by Ang-(1-7) to reduce tumor fibrosis. However, the inhibition of collagen synthesis by the heptapeptide may also participate in the reduction in breast tumor growth. Collagen I forms a tissue scaffold to promote tumor growth and stimulates expression of genes associated with cellular metabolism, transcription, and translation, including eukaryotic translation initiation factor 4B, glutamyl-prolyl-tRNA polymerase, and poly(A) polymerase, playing a role in both tumor structure as well as growth and progression (27). Intratumoral collagen I modulates E-cadherin-mediated cell-to-cell contact to increase tumor invasiveness and metastases (28). This suggests that the Ang-(1-7)-mediated decrease in collagen I production may also participate in the

reduction in tumor proliferation as well as the inhibition of tumor metastases.

Fibronectin, another component of the ECM secreted by activated myofibroblasts, is involved in cell-matrix and cell-cell adhesions, cell migration, and oncogenic transformation (29). The deposition of fibronectin in breast tumor stroma is positively correlated with tumor grade, size, and lymph node invasion (30). Fibronectin stimulates tumor cells through activation of integrin signaling to activate both focal adhesion kinases and MAPKs, increasing cell proliferation, survival, and angiogenesis (31). We observed a 40% decrease in fibronectin in Ang-(1-7)-treated isolated tumoral fibroblasts, suggesting that the heptapeptide may attenuate ECM deposition, tumor invasion, and proliferation by reducing fibronectin.

TGF- β binds to its receptors on fibroblasts to activate both MAPK and Smad signaling pathways and stimulate cell proliferation and fibrosis. Activation of the Smad signaling pathway by TGF- β results in ECM synthesis and deposition, tenascin-C production, as well as its own production, creating an autocrine cycle of fibroblast activation and ECM deposition (5). TGF- β 1 expression in breast cancer biopsies positively correlated with the rate of disease progression, independent of node status, tumor stage, age, and estrogen receptor status, suggesting its role in tumor progression (32). Because Ang-(1-7) reduced TGF- β 1 in isolated tumoral

⁴ In preparation.

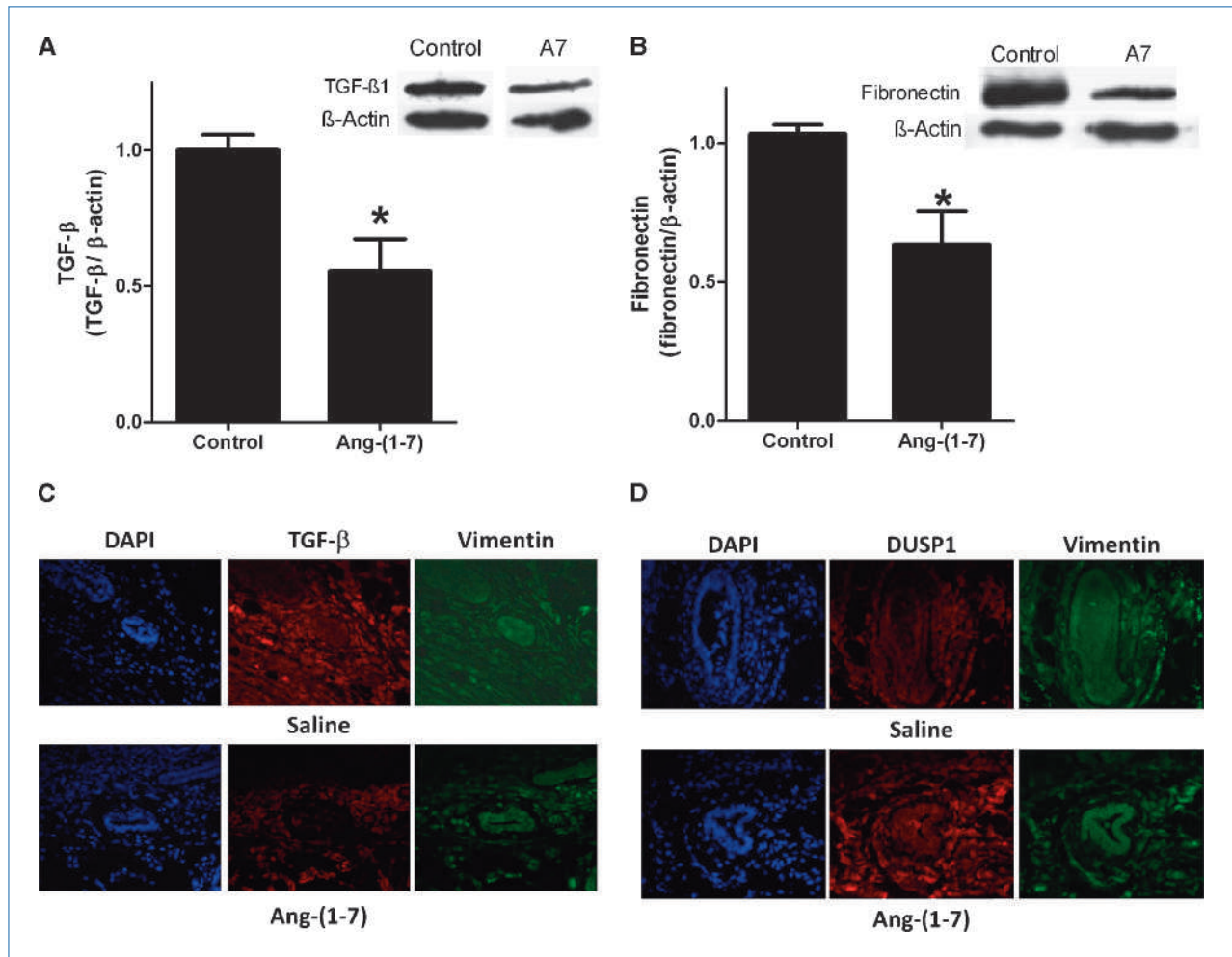


Figure 6. Effect of Ang-(1-7) on TGF- β , fibronectin, and DUSP1 in isolated tumor myofibroblasts and tumor sections. A, isolated tumor myofibroblasts were serum starved overnight and incubated with 100 nmol/L Ang-(1-7) (A7) for 24 h. TGF- β was assessed by Western blot hybridization and quantified as a function of β -actin. B, isolated tumor myofibroblasts were serum starved overnight and treated with 100 nmol/L Ang-(1-7) (A7) and 10 ng/mL TGF- β 1 for 24 h. Fibronectin was assessed by Western blot hybridization and quantified as a function of β -actin ($n = 3-4$). *, $P < 0.05$. C, ZR-75-1 tumors from mice treated with saline or Ang-(1-7) were incubated with antibodies to TGF- β and vimentin and counterstained with DAPI. Representative pictures are shown at $\times 200$ magnification. D, ZR-75-1 tumors from mice treated with saline or Ang-(1-7) were incubated with antibodies to DUSP1 and vimentin and counterstained with DAPI. Representative pictures are shown at $\times 200$ magnification.

fibroblasts, the heptapeptide may attenuate myofibroblast activation by reducing TGF- β 1 synthesis and signaling.

The exact molecular mechanism for the Ang-(1-7)-mediated downregulation of TGF- β 1 is not known. However, we previously showed that Ang-(1-7) increased cyclic AMP (cAMP) in VSMCs; (33) because an increase in cAMP inhibited TGF- β -stimulated collagen synthesis by ERK1/2 signaling in cardiac fibroblasts (34), Ang-(1-7) may increase cAMP in tumoral fibroblasts to reduce TGF- β . In addition, the DUSP1 promoter contains a cAMP-responsive element (35), suggesting that Ang-(1-7) may increase cAMP in tumoral fibroblasts to upregulate DUSP1, reduce MAPK activities, and inhibit fibrosis.

The amount of interstitial fibrosis was 3-fold higher in ZR-75-1 tumors than in BT-474 tumors, as assessed by picrosirius red staining in saline-treated mice. Several factors could account for this difference. BT-474 cells express the c-Met

receptor, whereas ZR-75-1 cells do not. Because activation of the c-Met receptor by HGF reduces fibrosis (36, 37), HGF signaling through the c-Met receptor may be responsible for the decreased total interstitial fibrosis observed in the BT-474 tumors (38, 39). On the other hand, HER2 signaling increases the expression of the Wilms' tumor suppressor gene (WT1) product through activation of protein kinase B (Akt; ref. 40). WT1 is implicated in tumor suppression and progression as well as inhibition of TGF- β signaling, which may reduce interstitial fibrosis (41, 42). The overexpression of HER2 in BT-474 cells and increased WT1 signaling could also contribute to reduced interstitial fibrosis. Further investigation is warranted to determine the molecular mechanism for the differences in interstitial fibrosis in the ZR-75-1 and BT-474 tumors.

We observed a significant reduction in perivascular fibrosis in BT-474 tumors from Ang-(1-7)-medicated mice when

compared with tumors from saline-treated mice. It was not possible to quantify perivascular fibrosis in the ZR-75-1 tumors due to pervasive interstitial fibrosis. The heptapeptide-mediated reduction in perivascular fibrosis in BT-474 tumors may lead to an overall decrease in the tumoral interstitial fluid pressure. High interstitial fluid pressure, found in breast tumors as well as in other types of tumors, is associated with vessel leakiness, lymph vessel abnormalities, and perivascular fibrosis, leading to matrix rigidity and fibroblast contractility and resulting in increased fiber tension (43, 44). This results in decreased transcapillary transport, limiting chemotherapeutic drug delivery to the tumor. These results suggest that Ang-(1-7) may enhance the delivery of chemotherapeutic agents when administered in combination with other therapeutic drugs (43).

The inhibition of tumor fibrosis by Ang-(1-7) is supported by previous studies showing the antifibrotic effect of Ang-(1-7) in cardiac and renal cells and tissues. Ang-(1-7) infusion prevented cardiac fibrosis in deoxycorticosterone acetate-salt-induced hypertension with a significant decrease in left ventricular wall fibrosis and reduced perivascular fibrosis (45), in agreement with studies showing that the heptapeptide reduced collagen formation and TGF- β in rat cardiac fibroblasts (46). Studies by our group showed that Ang-(1-7) infusion reduced cardiac fibrosis in Ang II-treated rats, further illustrating the role of Ang-(1-7) as an antifibrotic agent (47). Mice with ablated *mas*, the Ang-(1-7) receptor, have impaired cardiac function with increased cardiac collagen I, collagen III, and fibronectin deposition (48), whereas *mas* deletion increased collagen III, collagen IV, and fibronectin in the renal cortex and medulla (49). Our results show that Ang-(1-7), through activation of *mas*, also inhibits tumoral fibrosis.

In a recently reported phase I clinical trial assessing Ang-(1-7) as a chemotherapeutic agent, we showed that the heptapeptide reduced plasma placental growth factor in patients with clinical benefit (50), in agreement with our preclinical studies showing that Ang-(1-7) inhibits angiogenesis (16). Because activated myofibroblasts secrete cytokines that stimulate blood vessel formation (5), Ang-(1-7) may reduce angiogenesis by inhibiting the growth of tumor-associated fibroblasts. Taken together, these results suggest that Ang-(1-7) has pleiotropic effects on the tumor microenvironment to decrease tumor fibrosis, inhibit angiogenesis, and reduce tumor growth.

Disclosure of Potential Conflicts of Interest

P.E. Gallagher and E.A. Tallant hold a patent for the treatment of cancer with Ang-(1-7). The other authors disclosed no potential conflicts of interest.

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Katherine L. Cook, Linda J. Metheny-Barlow, E. Ann Tallant, et al.

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