Angiotensin-(1-7) Inhibits Growth of Human Lung Adenocarcinoma Xenografts in Nude Mice through a Reduction in Cyclooxygenase-2

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Abstract
Angiotensin-(1-7) [Ang-(1-7)] is an endogenous peptide of the renin-angiotensin system with vasodilator and antiproliferative properties. Our previous studies showed that Ang-(1-7) reduced serum-stimulated growth of human lung cancer cells in vitro through activation of a unique AT(1-7) receptor. The current study investigates the effect of Ang-(1-7) on lung tumor growth in vivo, using a human lung tumor xenograft model. Athymic mice with tumors resulting from injection of A549 human lung cancer cells were treated for 28 days with either l.v. saline or Ang-(1-7), delivered by implanted osmotic mini-pumps. Treatment with Ang-(1-7) reduced tumor volume by 30% compared with the size before treatment; in contrast, tumor size in the saline-treated animals increased 2.5-fold. These results correlate with a reduction in the proliferation marker Ki67 in the Ang-(1-7)–infused tumors when compared with the saline-infused tumor tissues. Treatment with Ang-(1-7) significantly reduced cyclooxygenase-2 (COX-2) mRNA and protein in tumors of Ang-(1-7)–infused mice when compared with mice treated with saline as well as in the parent A549 human lung cancer cells in tissue culture. These results suggest that Ang-(1-7) may decrease COX-2 activity and proinflammatory prostaglandins to inhibit lung tumor growth. In contrast, the heptapeptide had no effect on COX-1 mRNA in xenograft tumors or A549 cells. Because Ang-(1-7), a peptide with antithrombotic properties, reduces growth through activation of a selective AT(1-7) receptor, our results suggest that the heptapeptide represents a novel treatment for lung cancer by reducing COX-2.

Introduction
Lung cancer is a leading cause of death among men and women in developed countries, accounting for over 170,000 new cases and 160,000 deaths during 2004 in the United States (1, 2). Despite improvements in treatment modalities, the 5-year survival rate has improved to only 14% in the past 30 years, with over a million new cases of lung cancer diagnosed worldwide annually. This high mortality often is due to the presence of advanced-stage metastasis at the initial diagnosis, with more than two thirds of patients showing lymph node involvement at the time of presentation. This grim prognosis indicates a continued need for novel therapeutic approaches to reduce lung cancer mortality. In a retrospective study of over 5,000 patients in Scotland, the relative risks of incident and fatal cancer among patients treated with angiotensin-converting enzyme (ACE) inhibitors were significantly reduced, with the lowest relative risk in patients with lung or sex-specific cancers (3). ACE catalyzes the conversion of angiotensin I (Ang I) to the biologically active peptide Ang II as well as the breakdown of both bradykinin and the NH2-terminal heptapeptide fragment of Ang II [Ang-(1-7)] into inactive fragments (4, 5). Ang-(1-7), which is found endogenously at concentrations similar to Ang II, has both vasodilator and antiproliferative properties (6, 7). Because treatment of patients or animals with ACE inhibitors results in a significant elevation in the circulating and tissue levels of Ang-(1-7) (8, 9), we propose that the reduced lung cancer incidence in ACE inhibitor–treated patients may be due in part to the antiproliferative actions of the heptapeptide.

Ang-(1-7) attenuated vascular proliferation in vitro in cultured vascular smooth muscle cells (VSMC) and in vivo following vascular injury (7, 10–13). Ang-(1-7) also decreased protein synthesis in myocytes (14) and DNA and protein production in cardiac fibroblasts (14, 15), indicating that the heptapeptide regulates cardiovascular cell growth. Furthermore, Loot et al. (16) showed that an 8-week infusion of Ang-(1-7) in rats after coronary artery ligation improved cardiac function, which was correlated with a significant decrease in myocyte size in vivo. Thus, the reduction in DNA synthesis in vitro in VSMCs and myocytes as well as inhibition of neointimal growth and improvement of cardiac function observed in rats after Ang-(1-7) infusion show the antiproliferative effects of the heptapeptide.

Cyclooxygenases, key enzymes in the conversion of arachidonic acid to prostaglandins and thromboxanes, are important in the regulation of cellular growth and are altered under pathologic conditions, such as inflammation and tumor growth. Mitogen-inducible cyclooxygenase-2 (COX-2) is elevated in lung cancers when compared with nonmalignant tissue controls (17, 18). The increase in COX-2 is associated with increased production of prostaglandin E2 (PGE2), PGD2, and thromboxane A2 (TXA2), which are procarcinogenic and contribute to new vessel formation, angiogenesis, and tumor growth (19). In contrast, prostacyclin is a potent vasodilator and inhibits cell growth. Pulmonary-specific overexpression of prostacyclin synthase was associated with a significant reduction in tumor multiplicity in carcinogen-induced lung tumors in mice (20). Inhibition of COX-2 activity by treatment with selective COX-2 inhibitors attenuates the proliferation of malignant cells in vitro (18) and reduces tumor growth and metastasis (21, 22). These reports suggest that COX-2 plays a role in the pathology of lung cancer. Because the signal transduction mechanisms for the antiproliferative effects of Ang-(1-7) include changes in arachidonic acid metabolites and the enzymes involved in their production (7, 12, 23–25), a reduction in COX-2 and the...
associated decrease in PGE2, PGD2, and TXA2 or an increase in prostacyclin may participate in the antigrowth effects of Ang-(1-7).

In our previous study, we found that Ang-(1-7) inhibits the proliferation of human lung cancer cells in vitro (26). The anti-proliferative effect of the heptapeptide was blocked by the selective Ang-(1-7) receptor antagonist \([\text{d-alanine}^-]\)-angiotensin-(1-7). In the present study, we extend these studies to evaluate whether Ang-(1-7) inhibits lung tumor growth in vivo using a human lung xenograft model and to examine the role of COX-2 in the antitumorogenic properties of the heptapeptide.

Materials and Methods

Cell culture. A549 cells (CCL-185), obtained from the American Tissue Culture Collection (Manassas, VA), are lung adenocarcinoma cells derived from a 58-year-old male Caucasian. The cells were maintained at 37°C in a humidified atmosphere of 5% CO2 in Ham's F12 medium with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 units/mL streptomycin. All media and growth reagents were purchased from Life Technologies (Grand Island, NY).

Human xenografts. Male athymic mice (15–20 g, 2–4 weeks of age; Charles River Laboratory, Wilmington, MA) were housed in cages with HEPA-filtered air (12-h light/dark cycle) and \textit{ad libitum} access to food and autoclaved water. All procedures complied with the policies of the Wake Forest University Animal Care and Use Committee. Mice were inoculated s.c. in the lower left flank with \( \sim 1.9 \times 10^6 \) A549 lung cancer cells suspended in 200 μL of cold Matrigel (BD Biosciences, Bedford, MA). After 32 days, the mice were randomized for treatment with either saline or Ang-(1-7). The mice were anesthetized by inhalation with 1.5% isoflurane. An osmotic mini-pump (Alzet model 2004, Durect Corp., Cupertino, CA) was inserted into the peritoneum to infuse either 24 g/kg/h of Ang-(1-7) (Bachem, King of Prussia, PA) in saline or saline alone (6 mL over 28 days). The mini-pumps also contained heparin (25 units/mL) to maintain patency of the catheter. On day 28 of the infusion, the animals were anesthetized with halothane and sacrificed by decapitation.

Immunohistochemistry. Tumors were fixed with 4% paraformaldehyde for 24 h and incubated in 70% ethanol for 48 h before embedding in paraffin. The embedded tumors were cut into five micron thick sections and stained with H&E to determine morphology. Cell proliferation in the tumors was detected by immunostaining with an antibody to Ki67 (1:25; Abcam, Cambridge, MA) using the labeled streptavidin biotin method, as described previously (27). Stained sections were visualized and photographed with a computer-assisted counting technique with a grid filter to select cells was used to quantify the immunohistochemical staining of Ki67 (28). The positive cells were expressed as a percentage of the total cell number examined (100 cells sampled from each tissue site within each lung tumor section).

RNA isolation and reverse transcription/real-time PCR. RNA, isolated from cells or tissue using the TRIzol reagent (Life Technologies/Invitrogen, Carlsbad, CA), was subjected to reverse transcription/real-time PCR as previously described (14). All reactions were done in triplicate, and 18S rRNA, amplified using the Taqman rRNA Control kit (Applied Biosystems, Foster City, CA), served as an internal control. The results were quantified as \( C_t \) values, where \( C_t \) is defined as the threshold cycle of PCR at which amplified product is first detected and defined as relative gene expression (the ratio of target/control).

Western blot hybridization. Quiescent A549 lung cancer cells or tumor tissue cut into \( 1 \times 2 \) mm2 size pieces was homogenized in PBS [50 mmol/L NaPO4 (pH 7.2), 100 mmol/L NaCl]. Plasma membranes were isolated by centrifugation and solubilized by boiling in 3% SDS-10% \( \beta \)-mercaptoethanol. PBS was added to the membrane fraction to solubilize the proteins, and the protein concentration was measured by a modification of the Lowry method (29). Solubilized protein (20 μg per well) from saline- and Ang-(1-7)-treated animals was separated by SDS-PAGE and transferred to polyvinyl membranes (Amersham Pharmacia, Piscataway, NJ). Nonspecific binding was blocked with 5% blotto (5% evaporated milk, 0.1% Triton X-100) in TBS [50 mmol/L Tris-HCl (pH 7.4), 50 mmol/L NaCl]. The membranes were incubated with a COX-2 antibody (1:1,000; Cayman Chemicals, Ann Arbor, MI) followed by a goat anti-rabbit antibody (1:1,000; Amersham, Piscataway, NJ) coupled to horseradish peroxidase. Chemiluminescence reagents were added to visualize the immunoreactive bands, which were quantified by densitometry. An antibody to actin (Sigma, St. Louis, MO) served as the loading control.

Results

Inhibition of human lung cancer cell growth in vivo. The effect of Ang-(1-7) on tumor growth was examined in nude mice with human lung tumor xenografts. Athymic mice were injected with actively proliferating A549 human lung cancer cells in Matrigel. When the tumors were \( \sim 100 \) mm2 in size, at day 32, the animals were treated i.v. with either saline or Ang-(1-7) using an osmotic mini-pump. The first day of infusion was designated as day 0, and the animals were sacrificed after 28 days of treatment. Ang-(1-7) was administered at a dose of 24 μg/kg/h, based on our previous studies with rats showing that this infusion rate was well tolerated with no change in body weight, blood pressure, or heart rate and resulted in a 2- to 3-fold elevation in circulating Ang-(1-7) (10). During the infusion period, the animals maintained their body weight as well as food and water consumption and showed no evidence of reduced motor function. Additionally, no gross pathologic abnormalities were observed in major organs following sacrifice, indicating a lack of toxic side effects at the dose given.

No significant difference in the tumor volume of either group was observed before pump implantation before randomization for treatment with either saline (96.9 ± 14.4 mm3) or the heptapeptide (117.7 ± 21.7 mm3). With increasing time, tumors in the saline-treated mice continued to grow (Fig. 1), whereas tumor volume was arrested significantly in mice infused with Ang-(1-7). As shown in Fig. 24, Ang-(1-7) infusion resulted in a significant reduction in the

![Figure 1](cancerres.aacrjournals.org)
average tumor volume compared with the tumors in the saline-treated animals at the end of the 28-day infusion period [saline, 326.3 ± 47.2 mm³ versus Ang-(1-7), 84.4 ± 19.8 mm³; \( P < 0.05, n = 5 \)]. Moreover, a paired comparison of the tumor volume before and after treatment showed that all the tumors in Ang-(1-7)–medicated animals were significantly reduced in size when compared with the pretreatment tumor volume at day 0 (Fig. 2B).

In contrast, the tumor volume of every saline-infused animal increased over the treatment period. After 28 days, the tumor volume was reduced 30% in Ang-(1-7)–treated mice when compared with tumor size before heptapeptide infusion (Fig. 2C). Conversely, the tumor size increased 2.5-fold in the saline-treated animals during the course of 28 days compared with the initiation of saline infusion. At the end of the study, the mice were euthanized, and the tumors were removed and weighed. As shown in Fig. 3, the tumors from mice treated with Ang-(1-7) weighed 50% less than the tumors of mice infused with saline (0.13 ± 0.01 g versus 0.28 ± 0.03 g; \( P < 0.05, n = 5 \)).

**Effect of Ang-(1-7) on cell proliferation.** A portion of each tumor was fixed in formalin for histologic analysis, and 5-μm sections from mice infused with saline or Ang-(1-7) were stained with an antibody to Ki67. Ki67 served as a marker of proliferation, as it is present throughout all phases of the cell cycle in actively growing cells (in G1, S, G2, and M); in contrast, Ki67 is absent in quiescent cells (in the G0 phase). As shown in Fig. 4, cells in tumors from mice infused with saline showed abundant, robust staining for Ki67, indicating active cell proliferation. In contrast, cells in tumors from Ang-(1-7)–infused mice showed reduced immunoreactivity with the Ki67 antibody, suggesting a relative lack of cell division in the presence of the heptapeptide. The proportions of proliferating cells in tumors from saline-infused mice was higher than the proportion in tumors from Ang-(1-7)–infused mice (73 ± 2.9% compared with 49.5 ± 9.4%; \( P < 0.05, n = 5 \)).

**Effect of Ang-(1-7) on COX-2.** Western blot hybridization was used to compare the amount of COX-2 protein in tumors from Ang-(1-7)–infused mice compared with saline-treated mice. Ang-(1-7) significantly reduced COX-2 protein by 59% in the mice infused with Ang-(1-7) when compared with the animals infused with saline [saline, 0.99 ± 0.12 compared with Ang-(1-7), 0.41 ± 0.09; \( P < 0.05, n = 5 \)], as shown in Fig. 5A. Similarly, an 8-h treatment of A549 cells with 100 nmol/L Ang-(1-7) caused a marked decrease in COX-2 protein, as shown in Fig. 5B (\( P < 0.05, n = 4 \)). These results suggest that Ang-(1-7) may selectively decrease COX-2 activity and the production of proinflammatory prostaglandins.

Total RNA was isolated from tumor tissue of mice infused with saline or Ang-(1-7) and COX-2 mRNA was measured by reverse transcription/real-time PCR to identify the mechanism for the reduction in COX-2 protein. Treatment with Ang-(1-7) resulted in a 57% decrease in COX-2 mRNA in the tumor xenografts compared with treatment with saline (Fig. 6A; \( P < 0.05, n = 5 \)). COX-1 mRNA was not detected in the A549 lung tumors. Treatment with 100 nmol/L Ang-(1-7) also caused a significant reduction in COX-2.
mRNA in A549 cells at 2, 4, and 8 h of treatment (Fig. 6B). In contrast, there was no change in COX-1 mRNA in quiescent A549 cells treated with 100 nmol/L Ang-(1-7) at 2, 4, or 8 h (data not shown).

Discussion

The present study is the first demonstration of the in vivo efficacy of Ang-(1-7) in human lung A549 adenocarcinoma xenografts in athymic mice. Administration of the heptapeptide not only arrested tumor proliferation but also caused a significant reduction in tumor volume when compared with size before treatment. Before the administration of either saline or Ang-(1-7), the xenograft tumors were comparable in size. The tumors continued to grow in mice infused with saline, increasing ~2.5-fold at the end of the 28-day treatment period. In contrast, tumors from mice infused with Ang-(1-7) for 28 days decreased in size (~30%) compared with their size at the initiation of treatment. These results suggest that Ang-(1-7) prevents the proliferation of lung cancer cells in vivo and expand our previous in vitro studies showing that Ang-(1-7) caused a marked decrease in the serum-stimulated proliferation of A549 cells in tissue culture as well as an additional human adenocarcinoma cell line (SK-LU-1) and a squamous cell carcinoma cell line, SK-MES-1 (26).

Ang-(1-7) was administered to the mice using an osmotic minipump with an infusion rate of 24 μg/kg/h, based upon our previous studies showing that this rate of infusion resulted in a 2- to 3-fold increase in plasma Ang-(1-7) (10) and resulted in plasma levels similar to those obtained by treatment with an ACE inhibitor (8, 9). No toxic effects were observed in rodents infused with Ang-(1-7) at this rate, with no change in body weight, heart rate, or blood pressure. In agreement, Loot et al. (16) and Langeveld et al. (13) reported no side effects following infusion of the same dose of Ang-(1-7). Similarly, we observed no adverse reactions or gross pathologic abnormalities in the mice medicated with the heptapeptide. These data are consistent with the finding of no adverse side effects in toxicity studies of patients administered the heptapeptide as adjuvant therapy for cytopenia during chemotherapy (30). Taken together, these studies suggest that the heptapeptide is well tolerated, an important characteristic of a pharmacologic agent and a primary requirement for a chemopreventive agent.

However, further study at an increased dosage and for longer times is warranted. The negative slope in Fig. 2B, representing reduced tumor growth in mice treated with Ang-(1-7), suggests that a longer infusion time may result in a further decrease in tumor size.

In this report, we found decreased immunostaining of Ki67 and a reduced proportion of proliferating cells in tumor slices from mice treated with Ang-(1-7) compared with the saline-infused controls. These results suggest that the heptapeptide prevents progression through the cell cycle or the signaling pathways that regulate the cell cycle. This is in agreement with our previous in vitro studies showing that pretreatment of human SK-LU-1 lung cancer cells with 10 nmol/L Ang-(1-7) reduced serum-stimulated phosphorylation of extracellular signal-regulated kinase 1 (ERK1) and ERK2 (by 61% and 68%, respectively), enzymes whose activities are increased by mitogen treatment (26). Ang-(1-7) may either inhibit or down-regulate (a) ERK1 and ERK2 directly, (b) the mitogen-activated protein kinase (MAPK) kinases that phosphorylate ERK1 and ERK2, or (c) the MAPK kinase kinase that activates MAPK kinase. Alternatively, Ang-(1-7) may stimulate or up-regulate a MAPK phosphatase, which would result in a decrease in active MAPK.
Ang-(1-7) caused a significant reduction in COX-2 protein and mRNA in both A549 tumor xenografts and A549 cells in tissue culture, with no change in COX-1. COX-2 is overexpressed in 70% to 90% of adenocarcinomas (18, 31) and plays an important role in the pathology of lung cancer. Clinical trials with nonselective COX inhibitors, nonsteroidal anti-inflammatory drugs (NSAIDS), such as aspirin and indomethacin, show that attenuation of COX activity reduces the risk for lung cancer. Harris et al. (32) showed a 68% reduction in relative risk for lung cancer in patients administered NSAIDS. Epidemiologic studies also show an association between lung carcinoma risk and regular use of NSAIDs (33). Regular NSAID users (thrice per week or more for 1 year or longer) had decreased relative risks of lung carcinoma associated with a significant reduction in tumor multiplicity in carcinogen-induced lung tumors in mice (20). These results indicate the importance of the ratio of PGE2 (or TXA2) to PGI2 in tumorigenesis. We showed that Ang-(1-7) increased prostacyclin synthesis in rat, porcine, and rabbit VSMCs, and that inhibition of prostaglandin production using the nonspecific COX inhibitor indomethacin and subsequent prostacyclin-mediated activation of the cyclic AMP–dependent protein kinase prevented the Ang-(1-7)–mediated reduction in VSMC growth (7, 10–12). Infusion of Ang-(1-7) also increased prostacyclin production in salt-induced hypertensive rats (24). In contrast, TXA2 was suppressed by Ang-(1-7) infusion, showing that the heptapeptide differentially regulates PGI2 and TXA2. Thus, Ang-(1-7) may contribute to the inhibition of the growth of lung cancer cells or lung tumors by up-regulating or activating PGIS to increase prostacyclin or by reducing PGE2 production or increasing breakdown, to alter the PGE2 (or TXA2)/PGI2, or by effects on both enzymes.

The precise molecular mechanism for the transcriptional regulation of COX-2 by Ang-(1-7) is unknown. The COX-2 promoter region is complex, containing a large number of binding sites for inducible transcription factors. Nuclear factor-κB (NF-κB), a primary regulator of COX-2, is activated by Ang II (45). This suggests that the Ang-(1-7)–mediated reduction of COX-2 may occur through a down-regulation or inhibition of NF-κB, as the actions of the heptapeptide often oppose the physiologic functions of Ang II (6). Studies are ongoing to reveal the transcriptional regulators involved in the down-regulation of COX-2 by Ang-(1-7).

The mechanism by which COX-2 increases metastatic growth includes both the inhibition of apoptosis (46) and stimulation of angiogenesis (47). Nimesulide, a selective COX-2 inhibitor,
significantly reduced the production of PGE2, and induced apoptosis in 25% of tumor cells compared with controls (22). Celecoxib and SC-236, additional selective COX-2 inhibitors, also inhibited both tumor growth and angiogenesis (34). Previous studies showed that Ang-(1-7) inhibited angiogenesis in a murine sponge model, a technique representative of new blood vessel formation from preexisting blood vessels during wound healing (48). In the present study, the decreased volume of all Ang-(1-7)-infused tumors compared with their size before treatment initiation indicates that the heptapeptide either inhibits angiogenesis and a concomitant loss of nutrient supply or stimulates apoptosis, to attenuate tumor growth.

The attenuation of lung cancer growth by Ang-(1-7) treatment may provide the molecular mechanism for the observational studies showing a decreased risk of lung cancers in hypertensive patients administered ACE inhibitors (3, 49, 50). These medications are currently in widespread use for the treatment of hypertension and cause a significant elevation in both tissue and circulating Ang-(1-7) (8, 9). Ang-(1-7) exerts its antiproliferative effects through activation of the G protein–coupled receptor mas (14, 26), representing a unique mechanism of action distinct from other cell growth modulators. Taken together, our in vitro and in vivo results suggest that Ang-(1-7) inhibits lung cancer cell growth through activation of a unique angiotensin peptide receptor and may represent a novel therapeutic and/or preventive treatment for lung cancer by reducing COX-2. Thus, Ang-(1-7) could be administered singly, or a therapeutic modality combining the heptapeptide with other chemopreventive agents could be used to provide synergistic protection.

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References


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