The angiotensin-converting enzyme 2 in tumor growth and tumor-associated angiogenesis in non-small cell lung cancer

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Abstract. Angiotensin II (AngII) is a multifunctional bioactive peptide and previous studies have shown that the renin-angiotensin system (RAS) of both host and tumor are important in tumor growth and angiogenesis in lung cancer. Angiotensin-converting enzyme 2 (ACE2) is a newly identified component of RAS, with 42% amino acid homology to ACE. However, the expression and function of ACE2 in non-small cell lung cancer (NSCLC) are still unclear. In the present study, we analyzed ACE2 expression in NSCLC tissue by Western blot analysis and immunohistochemistry. AngII concentrations in the tissue homogenate were also detected using radioimmunoassay. We also examined the function of ACE2 by transducing A549 cells with MSCV-ACE2. We have shown for the first time that ACE2 expression decreased in NSCLC tissue in which AngII was higher than the matching non-malignant tissues. A concentration of 10^{-6} mol/l of AngII significantly increased expression of vascular endothelial growth factor a (VEGFa) and AT1-R and decreased ACE2 expression. We also found that overexpression of ACE2 may have a protective effect by inhibiting cell growth and VEGFa production *in vitro*. ACE2 may become a target of novel strategies to treat NSCLC.

Introduction

Lung cancer is the leading cause of cancer deaths worldwide (1) and 85% of lung cancers are non-small cell lung cancer (NSCLC). Despite improvements in treatment modalities, the 5-year survival rate for NSCLC has improved to only 14% in the past 30 years. This grim prognosis indicates a continued need for novel therapeutic approaches to reduce lung cancer mortality.

The renin-angiotensin system (RAS) is important in regulating cardiovascular homeostasis and blood pressure (2). Angiotensin II (AngII), a biologically active octapeptide in RAS, mediates its biologic effect by binding to two subtypes of receptors: type 1 (AT1-R) and type 2 (AT2-R), which belong to the G-protein-coupled receptor superfamily (3). Emerging data suggest that, in addition to systemically produced angiotensin, the tumor environment contains all RAS components necessary to produce angiotensin locally and local RAS system contributes importantly to tumor angiogenesis and tumor progression (4-6). A large scale clinical trial for hypertension demonstrated that inhibitors of angiotensin-converting enzyme (ACE) reduced mortality rates not only in cardiovascular diseases but also for malignant tumors (7). The relative risk was lowest in patients with lung or gender-specific cancers. Recent studies have associated ACE inhibitor use with a lower likelihood of history of cancer in patients with diabetes (8). Indeed, many studies show that captopril and other ACE inhibitors reduce NSCLC growth and angiogenesis (9,10,28). These findings suggest that the ACE-AngII-AT1-R pathway may be associated with NSCLC progression.

Angiotensin-converting enzyme 2 (ACE2) is a newly identified component of RAS, with 42% amino acid homology to ACE. Studies demonstrate moderate ACE2 expression in lungs of both humans (11) and mice (12), with high levels of ACE2 in kidney, heart, testis and small intestine in both species (12-14). In the human lung, immunostaining has localized ACE2 to endothelial and smooth muscle cells of large and small blood vessels, as well as to types I and II alveolar epithelial cells and bronchial epithelial cells (15,16). ACE2 catalyzes conversion of AngI to Ang-(1-9) (17,18). Moreover, ACE2 converts AngII to Ang-(1-7) (19), a peptide with vasodilator and anti-proliferative properties. Various *in vivo* studies strongly suggest that a major role of ACE2 is indeed the generation of Ang-(1-7) from AngII, and that its conversion of AngI to Ang-(1-9) is not normally of physiological importance (20-22). Gallagher and Tallant (23) showed

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that treatment with Ang-(1-7) resulted in dose- and time-dependent reductions in serum-stimulated DNA synthesis in NSCLC cell lines. Recent studies show that Ang-(1-7) can inhibit lung tumor growth *in vivo*, using a human lung tumor xenograft model (24). ACE2 thus provides an apparent mechanism to directly balance levels of AngII and Ang-(1-7) to modulate the pressor/mitogenic and depressor/growth inhibitory arms of RAS. However, the expression and role of ACE2 in NSCLC is still unclear.

Based on previous studies, we hypothesized that ACE2 plays an important role in tumor growth and angiogenesis and that its expression may decrease in lung cancer tissues compared to non-malignant tissues. In this study we clarified the expression of ACE2 and AngII in NSCLC. We also investigated the effects of AngII on lung cancer cells and examined the impact of overexpression of ACE2 on VEGF expression and cell proliferation *in vitro*. The results reported here may provide the basis for a novel and effective strategy to treat lung cancer.

Materials and methods

Tissue acquisition. Histologically confirmed NSCLC samples and matching non-malignant tissues (n=19) were obtained from patients who underwent surgical resection at Ruijin Hospital of Shanghai Jiaotong University School of Medicine between 2005 and 2007. Matched pairs of NSCLC specimens were dissected from tumors and from adjacent tumor-free (TF) tissues. Four of the patients were excluded for history of hypertension, renal disease or protein degradation. Tissue samples were frozen in liquid nitrogen for Western blotting and AngII assays. In addition, formalin-fixed paraffin-embedded tissue blocks containing 64 NSCLC samples, obtained from patients who underwent surgical resection between 2003 and 2004, were subjected to immunohistochemistry. All patients signed appropriate consent for tissue acquisition and study. The study was approved by the Hospital Ethics Committee.

Immunohistochemistry. ACE2 were detected in NSCLC sections and normal lung tissues. Briefly, samples were deparaffinized in xylenes and graded alcohols and rehydrated in Tris-buffered saline (TBS, pH 7.5). Following antigen retrieval in citrate buffer (0.01 M, pH 6.0) for 45 min at 120˚C, sections were rinsed in TBS, treated with 3% hydrogen peroxide in TBS, rinsed and incubated with 10% normal rabbit serum for 30 min. Sections were incubated in anti-ACE2 polyclonal primary antibody (1:50) overnight at 4˚C, rinsed with TBS, then incubated in anti-goat biotinylated IgG antibody (1:200 dilution) for 90 min at RT. After incubating in streptavidin-HRP (1:300) for 30 min at RT, sections were developed with 3, 3'diaminobenzidine (DAB) substrate and counterstained with hematoxylin. Scoring of NSCLC was performed by a histopathologist counting at least 500 tumor cells in 5 different visual fields for each specimen. Intensity of immunoreactivity (intensity score) was stratified and scored as follows: 1 for ≤25% of cells staining positive; 2 for 26-50% of cells staining positive; 3 for 51-75% of cells staining positive; and 4 for >75% of cells staining positive. An intensity score of ≥2 with at least 50% of tumor cells staining positive for ACE2 indicated tumor patients with high expression; <2 intensity score with <50% of tumor cells staining positive for ACE2 staining indicated tumor patients with low expression. The total score for each cellular compartment was obtained as the product of intensity and the percentage of staining.

AngII concentration detection. NSCLC tissues were weighed and cut into small pieces after washing. Homogenates (10% w/v) were prepared in a solution containing 50 mM phosphate buffer (pH 7.3). AngII concentration in the culture homogenate was measured using radioimmunoassay, carried out at the Ruijin Hypertension Institute Laboratory.

Protein isolation. NSCLC tissues and matching non-malignant tissues were mechanically homogenized in RIPA buffer containing 100 mg/ml phenylmethylsulfonyl fluoride. Confluent cell plates were washed three times with ice cold phosphate-buffered saline, then cells were scraped into 1 ml RIPA buffer. The RIPA buffer cell lysate suspension was then centrifuged at 13,000 rpm for 30 min. The supernatant was collected and protein concentration determined spectrophotometrically using the Bradford Protein assay (Bio-Rad Laboratories, Hercules, CA).

Cell culture and cell growth assay. A549 lung cancer cells (Shanghai Institute of Cells) were maintained in Ham's F12 medium with 10% FBS, 100 mg/ml penicillin and 100 U/ml streptomycin using media and growth reagents from Gibco (Gibco BRL, Grand Island, NY). Cell preparation was done in a room kept at 37˚C with a humidified atmosphere of 5% CO_2 and 95% room-air. To measure the influence of different concentrations of AngII and overexpression of ACE2 on cell growth, A549 cells ($3x10^3$) were seeded into a 96-well plate and then half were treated with AngII (10^{-9}-10^{-6} mol/l) for 72 h. A549 cells alone and those transfected with vector or MSCV-ACE2 were also plated in a 96-well plate. After 24 h incubation with 10% FBS, the cells were serum-starved for 24 h, then incubated in serum-free medium for different lengths of time, as indicated. Afterward, 10 μl of cell count kit-8 (CCK-8, Dojindo, Kumamoto, Japan) were added into each well and incubated for 3 h. The plates were read at 450 nm with a Safire 2 spectrophotometer (Tecan Group, Ltd, Männedorf, Switzerland). Experiments were carried out in triplicate and repeated at least three times.

Retroviral vector construct and transduction. The pcDNA3.1 vector containing human-ACE2 cDNA was kindly provided by Dr Paul McCray (University of Iowa, Iowa City, IA). They were ligated into the *Bgl*II/*Xho*I site of the pMSCV plasmid, which expresses human-ACE2. 293T cells were transfected with DNA (4 μg pMD-gag-pol, 4 μg pMD-VSVG and 4 μg retroviral vector pMSCV-ACE2 or pMSCV) using Lipofectamine 2000 reagent (Invitrogen Corporation, Carlsbad, CA) and VSV-G-pseudotyped viral supernatant fractions collected after 48 h were transduced into A549 cells using polybrene (Sigma Corporation, Cream Ridge, NJ) and centrifugation. Transduced cells were selected in the presence of 4 μg/ml puromicin. The stable transfectants were maintained in 2 mg/ml puromicin medium.

Cell cycle analysis using flow cytometry. A549 cells ($5x10^5$) were seeded into a 100-mm culture dish and pre-cultured for 24 h. A549 cells overexpressing ACE2 and control cells were serum starved for 24 h. The cells were then incubated in Ham's F12 medium with 10% FBS for 72 h. The cells were trypsinized, washed twice with PBS and suspended in 500 μl PBS containing 0.1% FBS for 15 min on ice. The cell suspension was mixed with 5 ml ice cold 70% ethanol and stored at 4˚C until analysis. On the day of analysis, cells were washed twice and re-suspended in 1 ml PBS containing 0.1% FBS. After incubation with RNase A (250 μg/ml) for 30 min and staining with propidium iodide (PI, 10 μg/ml) for 10 min, cell cycle analysis was conducted using the FACS system (BD Biosciences, San Jose, CA). Histograms were generated and cell cycle analysis carried out using Wind MDI 2.8 software (Joe Trotter, Scripps Research Institute, La Jolla, CA).

Enzyme-linked immunosorbent assay (ELISA). VEGFa levels were measured in the supernatants of A549 cells. After 24 h incubation with 10% FBS, A549 cells overexpressing ACE2 and control cells were serum-starved for 24 h. Cells were then incubated in serum-free medium for 24 h, the supernatant recovered and VEGFa levels determined by human VEGFa enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN).

Real-time quantitative reverse transcription analysis. ACE, ACE2, AT1-R and VEGFa mRNA expression were examined by real-time quantitative reverse transcription-based polymerase chain reaction (qRT-PCR) in A549 cells and those stimulated with AngII (10^{-7}, 10^{-6} mol/l) for 15 min. ACE, ACE2 and VEGFa mRNA expression were also examined in A549 cells overexpressing ACE2 compared with control cells. Total RNAs were extracted from cells by TRIzol reagent. RNA was treated with DNase (Promega, Madison, WI) and complementary DNA was synthesized using a cDNA synthesis kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Fluorescence (qRT-PCR) was performed with the double-stranded DNA dye SYBR-Green (PCR Core Reagents, PE Biosystems, Warrington, UK) using the ABI PRISM 7300 system (Applied Biosystems). The SYBR-Green assay contained 1 μl 10X SYBR-Green PCR buffer, 0.8 μl deoxynucleoside triphosphate (dNTP) mixture, 0.1 μl AmpErase UNG (1 U/μl), 0.05 μl AmpliTaq Gold DNA Polymerase (5 U/μl), 1.2 μl $MgCl_2$ (25 mM), 0.1 μl forward and reverse primer (20 μM), 1 μl cDNA and 5.65 μl double distilled H_2O. PCR was begun with one cycle at 50˚C for 2 min and 95˚C for 10 min and preceded by 45 cycles with denaturing at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. All data were analyzed using ABI PRISM SDS 2.0 software (PerkinElmer, Wellesley, MA). Using the ΔCt method, GAPDH was coamplified to normalize the amount of RNA added to the reaction and the data were subjected to cycling threshold analysis. PCR was repeated at least three times. The primers used in this study were as follows: ACE forward 5'-CCGATCTGGCAGAACTTC-3' and reverse 5'-GTGTTCCAGATCGTCCTC-3'; ACE2 forward 5'-CCACTGCTCAACTACTTTGAGCC-3' and reverse 5'-CTTATCCTCACTTTGATGCTTTGG-3'; AT1-R forward 5'-ATATTCCCCCAAAAGCCAAATCC-3' and reverse 5'-TCAACCTCAAAACATGGTGCAG-3'; VEGFa forwads 5'-GTTCGAGGAAAGGGAAAGGGT-3' and reverse 5'-GCGAGTCTGTGTTTTTGCAGG-3'; GAPDH forward 5'-ATTGCCGACAGGATGCAGA-3' and reverse 5'-GAGTACTTGCGCTCAGGAGGA-3'.

Western blot analysis. ACE2 protein expression in tumor tissues was studied by Western blot analysis. AT1 receptor and VEGFa protein levers in A549 cells was studied after treatment with or without AngII (10^{-7}, 10^{-6} mol/l) for 24 h. Expression of ACE2, AT1 receptor and VEGFa were also measured in A549 cells overexpressing ACE2 compared with control cells. After being treated with protein assay reagent (Bio-Rad Laboratories), soluble protein was separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA). ACE2 antibody (AF933, R&D Systems), AT1 receptor antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), VEGFa antibody (1:1000, Santa Cruz Biotechnology) and ß-actin monoclonal antibody (1:10000, Sigma Corporation) were used to detect ACE2, AT1 receptor protein, VEGFa protein and ß-actin protein respectively. The immunoreactive bands were visualized by ECL plus reagent (Amersham Biosciences, Piscataway, NJ). Expression of VEGFa protein in A549 cells were analyzed with Scion Image (Scion Corporation, Frederick, MD).

Statistical analysis. Data are presented as means ± SD. Student's t-test was used to compare the differences between two groups. The correlation between ACE2 expression and clinicopathological data was examined using Chi-squared analysis. Differences were considered statistically significant at $P<0.05$ (two-tailed). All statistical analyses were conducted using SPSS version 11.0 (SPSS Inc., Chicago, IL).

Results

Expression of ACE2 and AngII concentration in NSCLC and adjacent TF tissues. We carried out Western blot analysis to determine expression of ACE2. Equal concentrations of the isolated protein were electrophoresed on sodium dodecyl sulfate polyacrylamide gels. Blots were stripped and reprobed with ß-actin antibody to control for loading errors. As shown in Fig. 1A, ACE2 protein expression was examined in all 15 comparison samples, 11 of which (~73%) were lower in NSCLC tissues than in adjacent TF tissues. We also found that AngII concentration in the tissue homogenate of NSCLC was higher than in matching non-malignant tissues (Fig. 1B).

Localization and tmmunohistochemical expression of ACE2 in NSCLC tissues. As shown in Fig. 2, ACE2 was localized to the membrane and cytoplasm. Among 64 samples of NSCLC tissues, 19 (29.6%) showed high ACE2 expression (Fig. 2A and C) and 45 (70.3%) showed low expression (Fig. 2B and D). The relationship between ACE2 expression in NSCLC and clinicopathological factors is summarized in Table I. Statistical analysis indicated that the level of ACE2 protein expression correlated with clinical stage (P=0.011) and smoking status

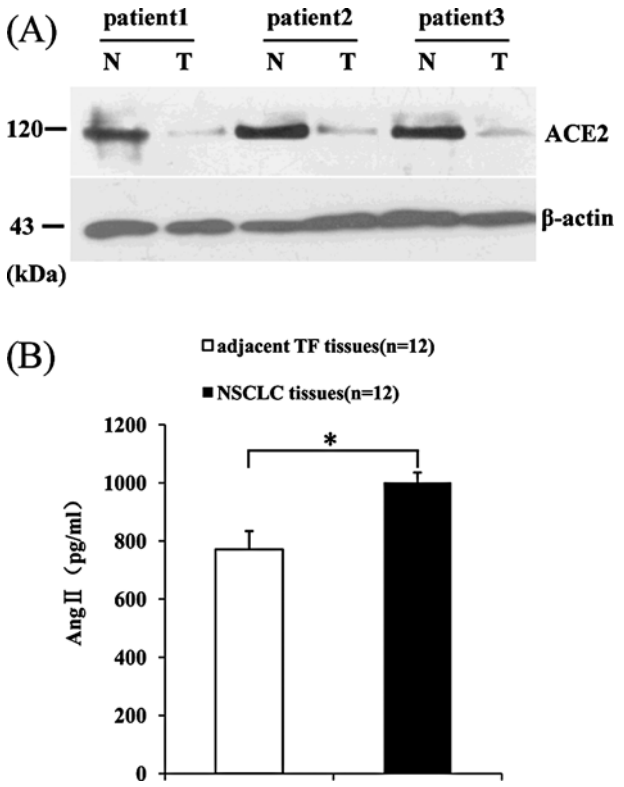

Figure 1. Expression of ACE2 and AngII concentration in normal and NSCLC tissue. (A) Representative Western immunoblots of protein extracts from three adjacent TF and NSCLC tissues showing expression of ACE2 with ß-actin as an internal control. N, adjacent TF tissue; T, NSCLC tissue. (B) AngII concentration in the NSCLC homogenate was measured using radio-immunoassay (means ± SD, n=12) *P<0.05.

Table I. Relationship between ACE2 protein expression and clinicopathological factors of patients with NSCLC.

Characteristics	Total	ACE2 protein expression		P-value
		High	Low	
Gender				
Male	42	11	31	0.398
Female	22	8	14	
Age at surgery				
≤55	32	6	26	0.055
>55	32	13	19	
Smoking condition				
Non-smokers	36	15	21	0.017
Smokers	28	4	24	
Tumor differentiation				
Well	20	9	11	0.157
Moderate	22	6	16	
Poor	22	4	18	
Clinical stage				
I-II	35	15	20	0.011
III	29	4	25	
Histological type				
Scc	32	7	25	0.171
Ad	32	12	20	

Scc, squamous cell carcinoma and Ad, adenocarcinoma.

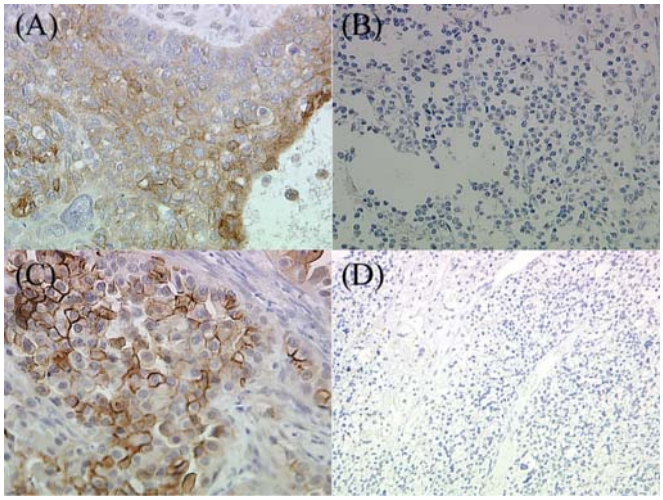

Figure 2. Immunohistochemical staining for ACE2 protein in NSCLC tissue. (A) High level of expression of ACE2 in squamous carcinoma tissue. (B) Low level of expression of ACE2 in squamous carcinoma tissue. (C) High level of expression of ACE2 in adenocarcinoma tissue. (D) Low level of expression of ACE2 in adenocarcinoma tissue (A, B, C, x400; D, x200).

(P=0.017), but expression groups did not obviously differ in gender, age, differentiation or histological type (SCC vs. AD).

Effect of AngII on VEGFa and RAS components in vitro. Analysis of (qRT-PCR) data of ACE, ACE2, AT1-R and VEGF mRNA, corrected with GAPDH as an internal control, showed that 10^{-6} mol/l of AngII significantly increased expression of VEGFa (Fig. 3A) and AT1-R mRNA (Fig. 3B, P<0.01) and decreased expression of ACE2 mRNA(Fig. 3C, P<0.01). In contrast, no change in ACE mRNA was observed in A549 cells treated with AngII (Fig. 3D), demonstrating the differential regulation of the two enzymes. In Western blot analysis, expression of VEGFa and AT1-R protein in 10^{-6} mol/l AngII-treated A549 cells were significantly higher than in 10^{-7} mol/l AngII and control cells (Fig. 3E and F).

Effect of ACE2 gene transfusion in A549 cells. Our objective was to establish the efficacy of MSCV-ACE2 transfusion. A549 cells were infected with MSCV-ACE2 and selected in the presence of puromicin. Infection of A549 cells with MSCV-ACE2 resulted in robust ACE2 expression by Western blot analysis at 72 h. No significant ACE2 protein expression was observed in A549 cells infected with the vector by the same way or in the normal lung tissue used as a positive control (Fig. 4A). As expected, expression of ACE2 mRNA (Fig. 4B, P<0.01) was notably higher in the MSCV-ACE2 group compared to vector. Moreover, AT1-R protein products (Fig. 4C) of A549 cells also decreased in the MSCV-ACE2 group compared to vector.

Effects of AngII and overexpression of ACE2 on cell growth of cultured A549 cells. Cell growth was determined by CCK-8 assay in A549 cells. As shown in Fig. 5A, treatment with

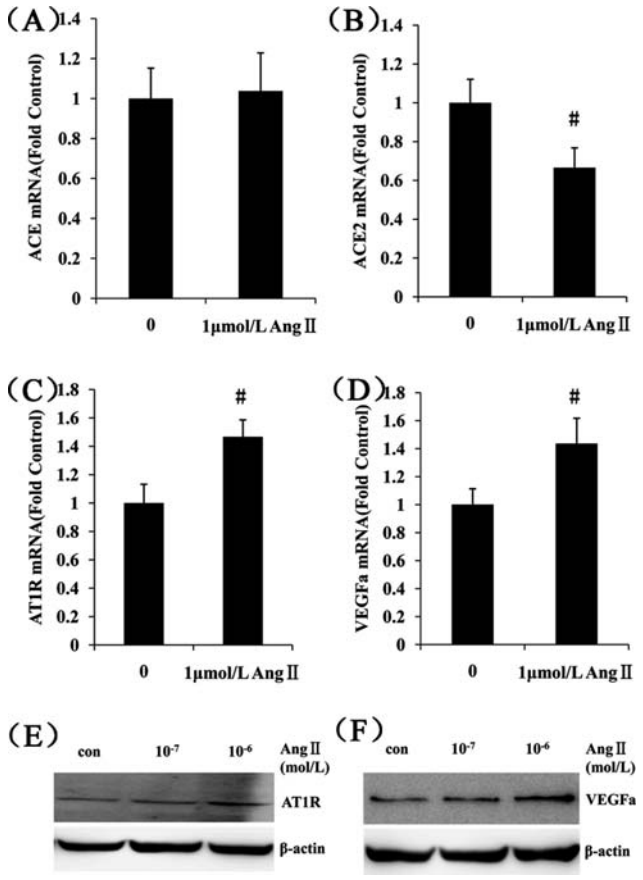

Figure 3. Effect of AngII on VEGFa and RAS components *in vitro*. (A, B, C and D). Effects of 10^{-6} mol/l of AngII on production of VEGFa mRNA (A), AT1-R mRNA (B), ACE2 mRNA (C) and ACE mRNA (D) in cultured A549 cells. (Means ± SD, n=3) #P<0.01. (E and F) Expression of VEGFa protein (E) and AT1-R protein (F) in cultured A549 cells with AngII treatment.

AngII (10^{-9}-10^{-6}) for 72 h did not affect viability of A549 cells. However, we found that ACE2 overexpression significantly decreased cell viability compared with vector (Fig. 5B). Furthermore, DNA staining by propidiumiodide was done to discriminate cells in G_0/G_1 or G_2/M phase by FACS (Fig. 5C). Overexpression of ACE2 induced a significant increase in cells in the G_0/G_1 phase and a decrease in cells in the G_2/M phase compared to vector. A549 cells infected with MSCV-ACE2 notably increased compared with vector in G_0/G_1 phase at 72 h (69.52±0.90% vs. 76.17±1.20%, P<0.01) and decreased in G_2/M phase at 72 h (9.33±0.87% vs. 5.62±0.58%, P<0.05) (Table II).

Table II. The effect of overexpression of ACE2 on cell cycle of A549 cells.

Group	Cell cycle distribution (means ± SD %)		
	G_0/G_1	S	G_2/M
Vector	69.52±0.90	21.16±0.06	9.33±0.87
MSCV-ACE2	76.17±1.20[b]	18.22±1.78	5.62±0.58[a]

[a]P<0.05; [b]P<0.01; compared with the vector group.

ACE2 inhibits AngII-induced VEGFa mRNA accumulation and protein production in cultured A549 cells. We sought to investigate whether overexpression of ACE2 can decrease VEGFa mRNA accumulation and protein secretion in A549 cells. Gene expression analysis by (qRT-PCR) showed that the VEGFa mRNA level decreased in A549 cells infected with MSCV-ACE2 compared with vector (Fig. 6A). VEGFa expression in A549 cells was detected by Western blot analysis. VEGFa protein levels in the supernatants were also determined by ELISA. As shown in Fig. 6B and C, VEGFa protein levels decreased in ACE2 infected A549 cells and supernatants compared with vector.

Discussion

The RAS system is important in regulating vascular homeostasis and AngII plays a role in proliferation, migration, and

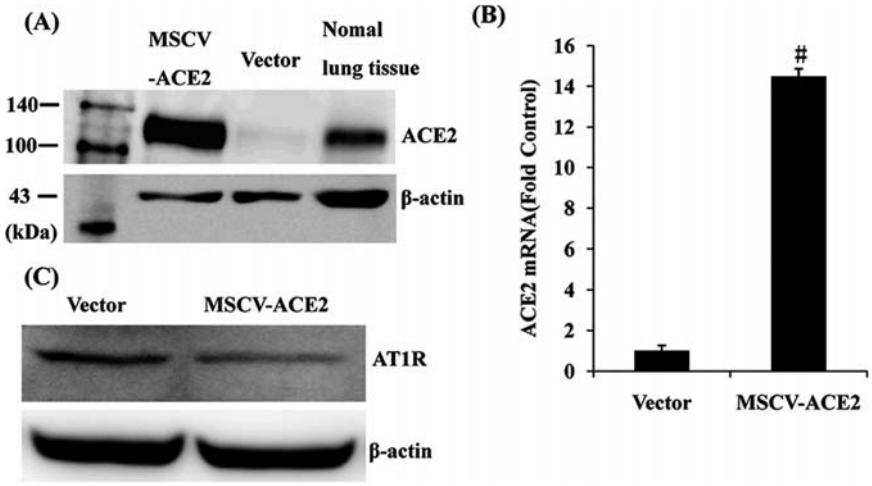

Figure 4. ACE2 expression and RAS components were measured in vector and MSCV-ACE2 groups in A549 cells. (A and B) ACE2 expression increased in MSCV-ACE2 group compared to vector by Western blot analysis (A) and real-time PCR (B) (Means ± SD, n=3) #P<0.01. AT1-R protein productions (C) of A549 cells decreased in MSCV-ACE2 group compared to vector. (Means ± SD, n=3) *P<0.05.

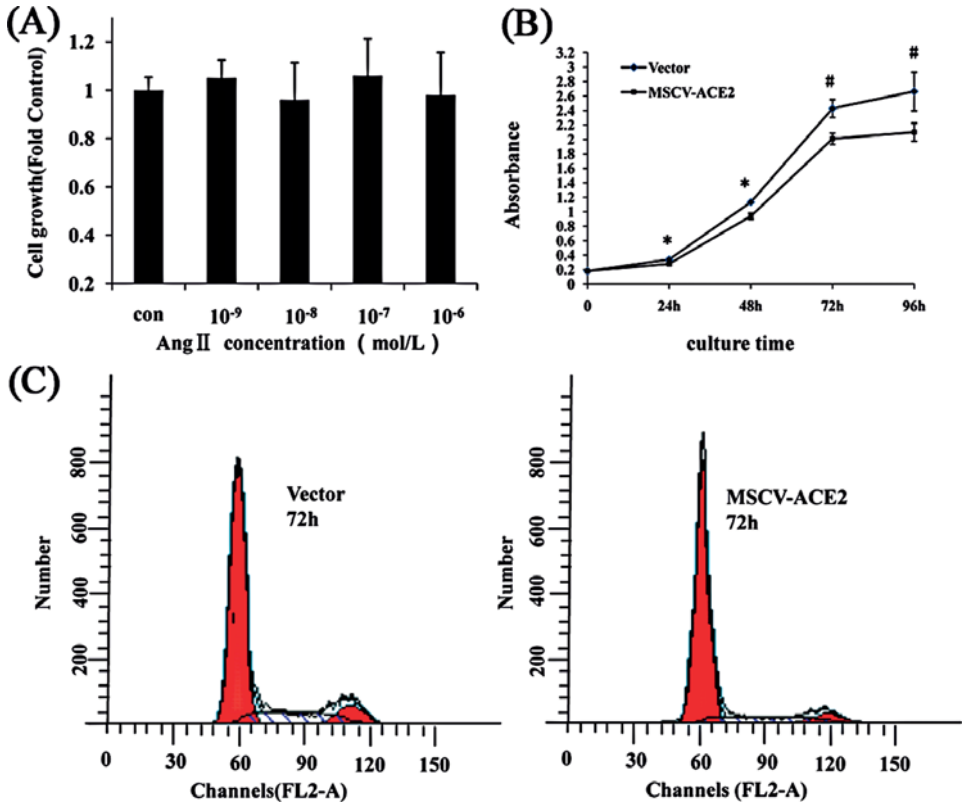

Figure 5. Overexpression of ACE2 reduced cell growth in A549 cells. (A) Treated with or without AngII (10-9-10-6) for 72 h did not affect cell viability in A549 cells. (B) Overexpression of ACE2 caused significantly decreased in cell viability. Cell growth was determined by the CCK-8 assay in A549 cells. Data are means ± SD values obtained from four culture wells per experiment, determined in three independent experiments. *P<0.05, $^{\#}$P<0.01, compared with the vector. (C) The FACS scans of cell cycle analysis of A549 cells infected with MSCV-ACE2. The A549 cells infected with MSCV-ACE2 and vector were incubated for 72 h. The cell cycle were analysed with flow cytometer using propidium iodide for DNA staining. This experiment was repeated separately three times. The results are expressed in the histogram.

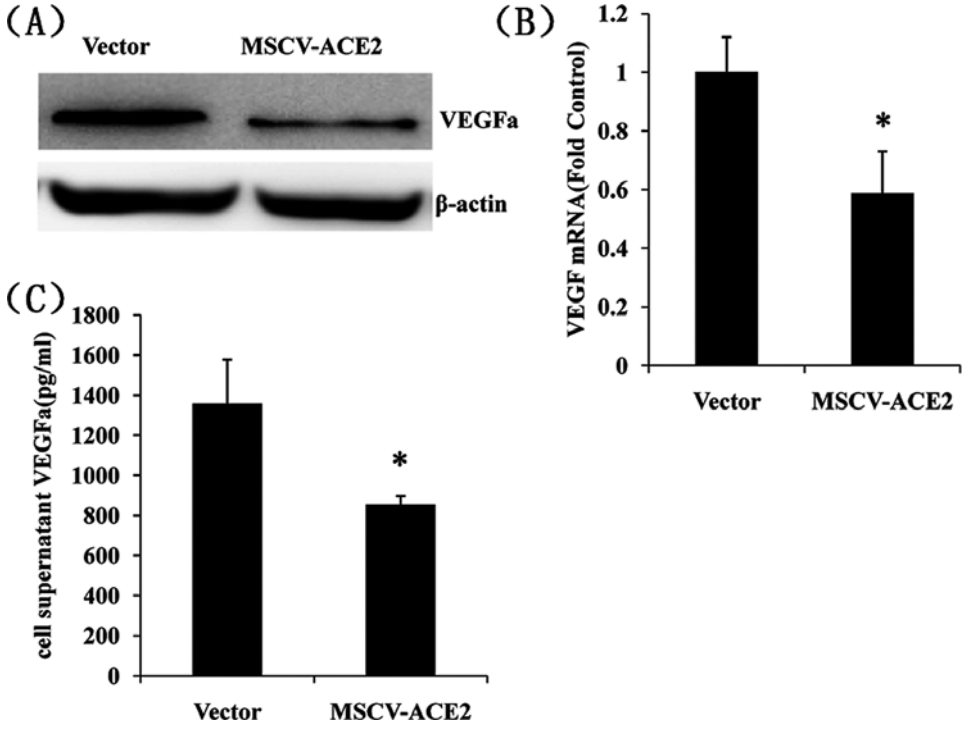

Figure 6. Overexpression of ACE2 inhibits AngII-induced VEGFa production. VEGFa production and mRNA accumulation decreased in A549 cells infected with MSCV-ACE2 compared with vector by Western blot analysis (A) and real-time PCR (B) (means ± SD, n=3) *P<0.05. (C) VEGFa protein levels in the supernatants were determined by ELISA. (Means ± SD, n=3) *P<0.05.

growth factor synthesis in several types of vascular cells. Expression of several components of RAS in various cancers, including brain, lung, breast, prostate, skin, cervix, and glioblastoma, has been previously demonstrated (25). Some studies show that the host stromal AT1 receptor pathway is important in tumor growth and tumor-associated angiogenesis (26,27). However, current evidence supports the idea that both host and tumor RAS are important in tumor growth and angiogenesis in lung cancer. Imai *et al* (28) found tumor size and VEGFa reduced significantly in $AT1a^{-/-}$ mice treated with TCV-116 (an AT1 receptor antagonist) compared with untreated mice. As a recently reported homologue of ACE, ACE2 is a new component of the updated RAS shown to be critical in the balance between AngII and Ang-(1-7) and to play a protective role in many diseases (29-31). However, its expression and effect in NSCLC remain unclear.

In this study, we demonstrated that ACE2 protein expression was lower in NSCLC tissues than in adjacent TF tissues by Western blot analysis. We also found higher AngII concentration in the tissue homogenate of NSCLC than in matching non-malignant tissues. In addition, immunohistochemical analysis showed that many NSCLC cells exhibited low to high ACE2 staining, mainly in the membrane and cytoplasm. Statistical analysis of ACE2 expression and the clinical features of patients with NSCLC showed that ACE2 expression was closely correlated to clinical stage and smoking status. This study is the first to investigate the expression of ACE2 and AngII in NSCLC. These findings suggest that the AngII-AT1-R system may play a significant role in the localized RAS within these NSCLC tumor tissues. Many situations influence RAS activity, including hypertension, renal disease and having taken ACEI or ARB. In our study, the 15 NSCLC tissue samples subjected to Western blot analysis were from patients with no history of hypertension, renal disease or taking ACEI or ARB.

Next, we examined whether AngII and overexpression of ACE2 could change the expression of other components of RAS. Consistent with a previous study (28), the main components of RAS were found to express in lung cancer cells. We found that expression of AT1-R protein in 10^{-6} mol/l AngII-treated A549 cells were significantly higher than in 10^{-7} mol/l AngII and control cells. Consistent with Western blot analysis results, (qRT-PCR) demonstrated an increase in mRNA expression of AT1-R. We also found that incubation of A549 cells with 10^{-6} mol/l AngII reduced ACE2 mRNA expression by (qRT-PCR). The AT1-R-mediated ERK/P38 MAP kinase signaling pathway may be a key mechanism by which AngII downregulates ACE2 expression (32). In contrast, AngII had no effect on ACE mRNA in A549 cells, demonstrating a differential regulation of the two enzymes by AngII. This finding is consistent with tests done in neonatal rat cerebellar and medullary astrocytes (33). Together, these findings demonstrated that AngII can magnify its effect in A549 cells through both decreased ACE2 production and increased AT1-R expression. This study provides the first evidence that overexpression of ACE2 decreases AT1-R production. This result suggests that ACE2 plays a important role in the local tumor RAS.

The roles of the AngII and AT1-R pathways in tumor progression, through effects on cellular proliferation, are unclear. AngII has been shown to stimulate proliferative and hypertrophic growth in vascular smooth muscle cells (34) and neonatal bladder stromal cells (35) through binding to AT1-R. However, it did not affect cell viability of murine Lewis lung carcinoma (LLC) cells (28) and pancreatic ductal adenocarcinoma (PDA) cells (36). We found that treatment with AngII (10^{-9}-10^{-6}) did not affect cell viability in A549 cells. Furthermore, consistent with the enhanced proliferative effect of ACE2 in PDA cells by RNA interference (37), we demonstrated overexpression of ACE2 inhibited cell growth compared to vector, even on the first day of the experiment. These data indicate that endogenously generated AngII is implicated in NSCLC cellular proliferation and viability. They also suggest that Ang-(1-7) may not inhibit early overexpression of ACE2, because Ang-(1-7) inhibits lung cancer A549 cell growth after five days (23). In the present study, cells in the G_0/G_1 phase significantly increased and those in the G_2/M phase decreased in MSCV-ACE2 cells compared to vector, indicating that ACE2 overexpression arrested growth in A549 cells.

As others have pointed out, VEGFa is an important mediator of angiogenesis (38). Consistent with previous studies in LLC cells, we found that VEGFa protein expression and VEGFa mRNA production were increased by stimulation with 10^{-6} AngII in A549 cells. Our results suggest that the tumor RAS promotes tumor angiogenesis through VEGFa induction. Kang *et al* (39) showed that AngII stimulated VEGFa synthesis in podocytes and that VEGFa production induced by AngII was mediated, in part, through activation of the p38 mitogen activated protein kinase pathway. Consistent with Western blot analysis and (qRT-PCR) results, we found VEGFa expression decreased in the supernatants of A549 cells infected with MSCV-ACE2 compared with vector. These findings reflect the fact that ACE2 inhibits tumor angiogenesis by decreasing VEGFa expression. Further studies will be required to discover the effect of ACE2 in tumor RAS using animal models.

In conclusion, the present study is the first to demonstrate lower expression of ACE2 in NSCLC tissues than in adjacent TF tissues and higher AngII concentrations in the tissue homogenate of NSCLC than in matching non-malignant tissues. We also demonstrated that ACE2 overexpression can decrease AT1-R protein production. Moreover, we showed that ACE2 may have a protective effect by inhibiting cell growth and VEGFa production *in vitro*. Combined with these findings, our data suggest that local RAS plays an important role in NSCLC development and progression. ACE2 may become the basis of a novel and effective strategy to treat NSCLC.

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