AngII induces HepG2 cells to activate epithelial-mesenchymal transition

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Abstract. The present study aimed to determine whether HepG2 can induce epithelial-mesenchymal transition (EMT) via angiotensin II (AngII) simulation. The expression levels of EMT markers vimentin and E-cadherin in cancer tissues and adjacent tissues of patients with hepatocellular carcinoma (HCC) were detected by immunohistochemistry. In addition, HepG2 cells were stimulated with AngII, and the gene and protein expression levels of vimentin and E-cadherin were measured by reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively, whereas cell migration and invasion were assessed using Transwell assays. The AngII inhibitor Angl-7 and the Angl-7 inhibitor A779 were added to the system to further evaluate AngII-induced EMT. Compared with that in normal tissue, the expression level of vimentin in HCC tissue was increased, whereas that of E-cadherin was decreased. EMT occurred 48 h following AngII stimulation. The transcription level of E-cadherin in HepG2 cells was decreased, whereas that of vimentin was increased. In addition, the migration and invasion abilities of the cells were increased simultaneously. Angl-7 partly inhibited AngII-induced EMT. When stimulated at an appropriate time, HepG2 cells have the ability to undergo EMT.

Introduction

Angiotensin-converting-enzyme (ACE) inhibitor (ACEI), angiotensin II receptor blocker (ARB) (1,2), and angiotensin II (AngII) inhibitor Angl-7 ameliorate tumor growth, metastasis and angiogenesis. The low cost, safety, and abundant clinical use of these substances have encouraged investigation of the association between the rennin-angiotensin system (RAS) and tumor metastasis (3).

In 1998, Lever et al (3) performed a retrospective analyzed 5,207 tumor cases and found that the long-term use of ACEI lowered the risk of breast and lung tumors. In the last 10 years, there has been continued investigation of the mechanisms of RAS in different types of tumor, including those in breast and cervical cancer, blastoma, hepatocellular carcinoma (HCC), and in the gastrointestinal tract (3). Studies have confirmed that AngII promotes tumor cell proliferation, angiogenesis, apoptosis and metastasis, whereas Angl-7 exerts the opposite effects. Inhibition with ACEI and ARB can delay tumor growth and prolong patient survival rate (4,5). The antagonist Angl-7 has entered its second clinical trial and may be available for clinical use in the future (6). However, owing to the complexity of RAS components, current knowledge of the mechanisms underlying their effects on tumor metastasis is limited, and the results of clinical trials remain controversial. It has been found that ARBs can increase the risk of tumors (7). Therefore, in-depth laboratory experiments in cell lines and animals are required involving RAS components, particularly AngII, which performs a key role in the system, to support its use in clinical trials.

Epithelial-mesenchymal/mesenchymal-epithelial transition (EMT/MET) is crucial in tumor metastasis and relapse, as liver cancer cells migrate through this process. AngII is important in the EMT of renal tract epithelial cells. However, few studies have focused on the roles of AngII inhibitors, ACE2, or Angl-7 in EMT/MET. The present study focused on AngII-induced EMT/MET in HepG2 cells.

Materials and methods

Materials. The present study was approved by the Institutional Ethics Committee of the Beijing University Shenzhen Hospital (Shenzhen, China; no. 2014024). All procedures performed in investigations involving human participants were in accordance with these ethical standards. Informed consent was obtained from all individual participants included in the study. AngII, Angl-7 and A779 were purchased from Sigma-Aldrich; EMD
Millipore (Billerica, MA, USA). Rabbit anti-human monoclonal GADPH antibody (cat. no. NB300-221), E-cadherin (cat. no. NB2-19051), and vimentin (cat. no. NB1-92687) antibodies were purchased from Novus Biologicals, LLC, Littleton, CO, USA. The HepG2 cell line, obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China) was used in the present study.

**Immunohistochemical analysis.** The human HCC tissue microarray used in the present study comprised 51 primary HCC (age range, 45-67 years; 40 males and 11 females) and six normal adjacent liver samples (3 cm away from the cancer tissue used for specimens). The HCC samples were collected from the patients between January 2012 to December 2013 in Beijing University Shenzhen Hospital during surgery. The patients, were informed at the time of collection and their consent was obtained with a signed informed consent form. Of the 51 HCC samples, 32, 9, 5, and 5 samples were classified as stages I, II, III, and IV, respectively. Following deparaffinization, the sections were permeabilized with a 0.1% TritonX-100 solution in PBS for 30 min. The sections were then blocked for 1 h at room temperature with 2% goat serum (Shanghai Haoran Biotechnology Co., Ltd., Shanghai, China) and 1% BSA (Shanghai Haoran Biotechnology Co., Ltd.) in PBS and then incubated with anti-E-cadherin antibody (1:2,000, mouse anti-human, Novus Biologicals, LLC) and anti-vimentin antibody (1:2,000, mouse anti-human, Novus Biologicals, LLC) overnight at 4°C. The sections were rinsed in PBS and then incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody (1:1,000; cat. no. 644001; Neobioscience; Shenzhen Xinboxsheng Biotechnology Co., Ltd., Shenzhen China) for 1 h at room temperature. The signals were developed by avidin-biotin-peroxidase complexes with a DAB substrate solution. The integrated optical density was calculated to analyze the semi-quantitative expression of E-cadherin and vimentin using Image-Pro plus 6.0 software (Media Cybernetics, Inc, Rockville, MD, USA). Two stained sections were randomly selected for each sample, with 10 different areas for each section.

**Cell culture.** The HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma; EMD Millipore) containing 100 U/ml of penicillin, 100 µg/ml of streptomycin and 15% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO2. The cells were synchronized in FBS-reduced medium (0.5%) for 24 h prior to the experiments. Normal-glucose DMEM was prepared with 5.5 mM of D-glucose, whereas high-glucose medium was prepared by supplementing normal DMEM with D-glucose for a final D-glucose concentration of 30 mM. Normal-glucose DMEM supplemented with 24.5 of mM mannitol served as an osmotic control in the experiment. AngI-7, which is a key component of RAS, represents one of the most significant conceptual changes of this important hormonal system and antagonizes the action of Ang II, or Mas receptor antagonist A-779 was applied 30 min prior to high-glucose treatment.

**Western blot analysis.** The cells were lysed in a buffer containing 50 mM Tris-Cl, 1% (w/v) SDS, sodium pyrophosphate, β-glycerophosphate, sodium orthovanadate, sodium fluoride, EDTA and leupeptin (Beyotime Institute of Biotechnology, Jiangsu, China). A protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) was supplemented prior to use. Equal quantities of protein (30 µg) were run on a 10% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked in 5% skim milk in Tris-buffered saline and 0.1% Tween 20 (TBST) for 1 h at room temperature. The membranes were then incubated with primary antibody (1:1,000 for mouse anti-vimentin, 1:1,000 for mouse anti-E-cadherin, 1:2,000 for rabbit anti-human GADPH) at 4°C overnight. Following three washes in TBST, the membranes were incubated in horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. 19468, Rockland Immunochemicals Inc. Limerick, PA, USA) for 2 h at room temperature. Hybridizing signals were detected using an enhanced chemiluminescence detection kit (Pierce; Thermo Fisher Scientific, Inc.) and then normalized to GADPH. Signals were qualified using an image-analysis software program (Smart View, Puri Technology, Shanghai, China).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** RT-qPCR analysis was performed using the Bio-Rad iCycler iQ RT-q PCR detection system (Bio-Rad Laboratories, Inc.). Total RNA from HepG2 cells was extracted using a TRIzol kit (Invitrogen; Thermo Fisher Scientific, Inc.). Absorbance was tested at 260 and 280 nm using a UV spectrophotometer to determine the level of RNA purity. The PrimerScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to perform reverse transcription and 10 µl of the total system provided by the kit was used for each reaction. SYBR Green Real-Time PCR Master mix was used to quantify the relative abundance of the target mRNA. The primers used for RT-qPCR are shown in Table I. The reaction conditions were as follows: A single cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The relative quantification of gene expression was normalized against actin, and the 2^(-ΔΔCt) method was used to represent the data (8). Each sample was run and analyzed in triplicate. The samples from the control group were used as calibrators with a given value of 1, and the conditioned groups were compared with the calibrator.

**Invasion assays.** Invasion was measured using 24-well BioCoat cell culture inserts with a polyethylene terephthalate membrane coated with Matrigel basement membrane matrix (100 µg/cm²; BD Biosciences, Franklin Lakes, NJ, USA). In brief, Matrigel was allowed to rehydrate for 2 h at room temperature by adding warm, serum-free DMEM. The wells of the lower chamber were filled with medium containing 5% FBS. The cells (5x10^4) were seeded in the upper compartment (6.25-mm membrane size) in serum-free medium. The invasion assay was performed at 37°C in a 5% CO2 humidified incubator for 22 h. At the end of the invasion assay, the filters were removed, fixed, and then stained with the Diff-Quick staining kit. Cells on the upper surface of the filters were removed by wiping with a cotton swab, and invasion was determined by counting the number of cells that had migrated to the lower
side of the filter under a microscope at x100 magnification. For each sample, 10 fields were selected to count the number of cells and calculate the average. The cells were treated with AngII (1x10⁻⁵ mmol/l), AngII+Ang1-7 (1x10⁻⁵ mmol/l), AngII+Ang1-7+A779 (1x10⁻⁵ mmol/l), Ang1-7+A779, or A779 at 37°C for 48 h in a 5% CO₂ incubator prior to plating on the invasion chamber. The cell density for this experiment was required to reach a confluence of 80% prior to treatment.

Statistical analysis. Data are presented as the mean ± standard error of the mean and were analyzed for statistical significance using one-way analysis of variance followed by Newman-Keuls test as a post hoc test. All statistical analyses were performed in SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) for Windows.

Results

Immunohistochemical assessment. Compared with that in normal tissue, the expression level of the EMT marker protein vimentin was significantly higher in the tumor center cells than in parietal cells, whereas the expression level of E-cadherin was significantly lower in the tumor cells (P<0.001; Fig. 1A-D, Table II).

Effect of AngII and the appropriate stimulating time of AngII to induce EMT. The results of the western blot analysis showed that the level of E-cadherin in the cultured HepG2 cells differed significantly from that in the control group (0 h) following stimulation for 24, 48, 72, and 96 h (P<0.05). The level of E-cadherin decreased whereas the level of vimentin increased in the group stimulated with AngII, compared with that in the group without drug administration, indicating that the cells cultured for 48 h following AngII stimulation underwent EMT. Therefore, the 48 h time point was selected to examine EMT (Fig. 2A and B; Table III).

Effects of Ang1-7 treatment on HepG2 EMT following AngII stimulation. Compared with the control group, the AngII-stimulated cells exhibited decreased E-cadherin and increased vimentin (P<0.05). Following AngII+Ang1-7 treatment, the expression levels of E-cadherin and vimentin did not differ significantly compared with those in the control group. This result indicated that the inhibitory effect of Ang1-7 on AngII stimulation was partly inhibited by A779. The A779, Angl-7, and Angl-7+A779 groups did not differ significantly from the control group (Fig. 3; Table IV).

Table I. Sequences of primers for reverse transcription-quantitative polymerase chain reaction analysis.

| Gene          | Primer sequence                  |
|--------------|----------------------------------|
| E-cadherin   | Forward 5'-CAGCACGTACACAGCCCTAA-3'  |
|              | Reverse 5'-ACCCACCTCTAAGGCGACCTCT-3'  |
| Vimentin     | Forward 5'-CGCACATTTGCAGCAAAGACA-3'  |
|              | Reverse 5'-GAGGGCTTCTAGCGTTTCTG-3'  |
| β-actin      | Forward 5'-GGAAGGTGGACAGGAGGCC-3'  |
|              | Reverse 5'-GTGACGTGGACATCACCAGAAG-3'  |

Table II. Protein expression of vimentin and E-cadherin, determined by immunohistochemical analysis.

| Sample               | Vimentin | E-cadherin |
|----------------------|----------|------------|
| Liver                | 0.0420±0.00040 | 0.0604±0.00024 |
| Tumor center         | 0.0876±0.00063 | 0.0327±0.00064 |
| T-value              | -173.643 | 53.357 |
| P-value              | <0.001 | <0.001 |

Effect of AngII stimulation on the gene expression of E-cadherin and vimentin in HepG2 cells. The relative RNA expression levels of E-cadherin and vimentin, which are the main genes expressed in HCC EMT, were measured using RT-qPCR analysis. The results revealed that E-cadherin was decreased in the AngII stimulation group, compared with that in the control group (P<0.05). No significant difference was observed between the AngII+Ang1-7 group and the control group, which was consistent with the results of the western blot analysis (Fig. 4).

Migration assay. The number of HepG2 cells that transferred through the wells (8.71±2.09) in the AngII stimulation group exceeded that of the control group (5.08±0.88). The number in the AngII+Ang1-7+A779 group (7.00±0.69) was significantly higher than that in the AngII+Ang1-7 group (6.72±1.69) but was significantly lower than that in the AngII group (Fig. 5A-G).

Discussion

Ang in the liver is converted into AngI, which is converted into Ang1-7 under the effect of ACE2. AngII acts through AT1, forming the ACE-Ang-AT1 axis; Angl-7 acts through MAS, forming the ACE2-Ang1-7-MAS axis. The two axes act with each other, modulating human physical functions (9,10).

It has been suggested that the main function of RAS is to modulate blood pressure and water-salt balance. Previous clinical studies and animal experiments have revealed that RAS is expressed abnormally in tumors and is associated with disease prognosis (11). RAS antagonists can prohibit tumor growth, metastasis and blood vessel formation. Retrospective clinical studies have shown that that the long-term administration of ACEI can prevent tumor occurrence, and this medication does not affect blood pressure (4,5). Earlier studies examined clinical samples, comparing the center of the tumor tissue with its adjacent tissue to identify differences in RAS expression. Their results showed that the expression levels of ACE1, ACE2, and AT1 in the tumor centers increased or decreased consistently, compared with those in adjacent tissues (7,10). These findings showed that the ACE1-AngII-AT1 and ACE2-Ang1-7-MAS axes acted with each other in patients with HCC. When ACE1 was increased, ACE2 increased in compensation and vice versa, reaching a certain balance. When ACE1 was increased, AT1 increased simultaneously and vice versa, indicating that ACE1 acted with AT1 (12,13).

The reason for AngII and its receptor induce the formation of blood vessels and tumor metastasis, and why ACEI
and ARB can inhibit tumor occurrence, proliferation, blood formation and metastasis remain to be fully elucidated (14). Studies have shown that ACEI or ARB drugs can inhibit the matrix metalloproteinase (MMP) family, particularly...
MMP2 and MMP9, whose main functions are to degrade base membrane construction-collagen type IV (15). ACEI and ARB can also inhibit vascular endothelial growth factor, and the latter can inhibit tumor occurrence and proliferation, which can be considered as mechanisms of tumor inhibition (16). Experiments using rats with AT1R -knockout found that AngII promotes blood vessel proliferation mainly on mesenchymal cells and not in the tumors themselves. Previous studies found that the more malignant epithelial originated cells exhibit more irregular expression of AngII and AT1R, and lower levels were correlated with the morphological changes of tumor cells (11,17). AngII induces transforming growth factor -β (TGFβ), a potent driver of cancer progression through the induction of EMT, in which epithelial cells acquire a mesenchymal phenotype, leading to enhanced motility and invasion. This process involves the activation of extracellular signal-regulated kinase, small mothers against decapentaplegic (Smad)2, and subsequently the TGFβ signaling pathway, promoting EMT and migration and invasion of human HCC cells (11). These findings may be explained using EMT theory.

Under specific circumstances, mature cells can exhibit plasticity, transferring from one phenotype to another. By interacting with the surrounding mesenchyme, these epithelial cells may lose certain epithelial characteristics, including inter-cell conjunction and polarity, and acquire several mesenchymal characteristics, including invasion and migration. This phenomenon is termed EMT (18,19). For convenience in investigating EMT, the 2007 International EMT Meeting (3) divided EMTs into three types according to the different surroundings in which EMT occurs: Type I EMT occurs during fertilized ovum implantation, fetus development and organ formation; type II EMT occurs during trauma healing, tissue reconstruction and fibrosis; and type III EMT transpires during tumor invasion and metastasis. Primary tumor site epithelial cells transform into mesenchymal cells through EMT, invading the basement membrane and transferring via blood vessels to form distant secondary tumor sites through MET (20). Microcircumstances have...
been considered important in tumor metastasis in previous years, and local RAS, which is expressed in the matrix, may be involved (21).

The present study confirmed that AngII induced EMT, with an appropriate time point of 48 h. The promotion effect appeared to be inhibited partly by Ang1-7, whereas the Ang1-7 inhibitor A779 partly ameliorated the inhibition. In order to obtain more stable experimental results, more detailed experimental protocols are to be designed in subsequent investigations, including the use of reagent concentrations and incubation time. These findings indicated that RAS components coordinated with each other to modulate tumor metastasis. A previous study (22) demonstrated that the ACE1-AngII-AT1 and ACE2-Ang1-7-MAS axes interacted with each other to modulate HCC.

Other associated studies have found that RAS can modulate tumor growth and metastasis through the TGF-β and Smad signaling pathways. Further investigations of the signaling pathways and in vivo experiments are required.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.
Authors' contributions

MQ and YZ completed the experimental design, performed and data analysis and wrote the paper. JL, XO and ML performed the experiments and contributed to the data analysis. XL, JY and GY collected the specimens. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of the Beijing University Shenzhen Hospital (no. 2014024). All procedures performed in investigations involving human participants were in accordance with these ethical standards. Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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