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Original Articles

Angiotensin II type 1 receptor antagonists inhibit cell proliferation and angiogenesis in breast cancer

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ABSTRACT

Angiotensin II type 1 receptor (AT1R) promotes tumor invasion, migration, metastasis and angiogenesis. We explored the potential antitumor effects of AT1R antagonists in breast cancer. We found that angiotensin II promoted cell proliferation and upregulated the expression of vascular endothelial growth factor A (VEGF-A) in MCF-7 cells. Losartan downregulated the expression of VEGF-A in MCF-7 cells treated with angiotensin II. Candesartan downregulated the expression of VEGF-A in mice bearing MCF-7 xenografts and inhibited tumor growth and angiogenesis. AT1R and VEGF-A expression correlated with increased microvascular density in 102 breast cancer patients. Our data suggest that AT1R antagonists might be useful to suppress breast cancer by inhibiting the angiotensin II.

1. Introduction

The incidence of breast cancer has been increasing gradually and now is considered a major threat to women's health [1,2]. Current breast cancer therapy consists of surgery and adjuvant postoperative local or systemic radiotherapy, chemotherapy and hormonal therapy [3,4]. Breast cancer is actually a collection of diseases each with distinct histopathological features, genetic and genomic variability, and different prognostic outcomes. As such, no single model completely recapitulates this complex disease. The advent of personalized medicine has the potential for profound effects on breast cancer research and treatment as its aims are to identify the specific events that drive development and progression of an individual's cancer and identifies patient specific therapeutic targets. Here we focus on angiotensin receptor II type 1 (AT1R) a potential molecular target in breast cancer therapy.

Angiotensin II (AT2), one of the major components of the renin-angiotensin system, is responsible for regulation of blood pressure and electrolyte balance by binding to AT1R and AT2R [5,6]. AT2 is a potent vasoconstrictor that is converted from angiotensin I by angiotensin-converting enzyme (ACE). Interestingly, women carrying a low-activity genotype of the ACE gene have been found to have a 50% reduction in the risk of breast cancer [7]. Oral ACE inhibitors (ACEIs, a class of antihypertensive drugs) have also been shown to have protective effect against breast cancer [8]. Moreover, ACEIs inhibited tumor growth and angiogenesis in mouse models of breast cancer [9].

Recent studies have shown that AT1R overexpression frequently occurs in a variety of tumors, and correlates with tumor angiogenesis [10–13]. Marked AT1R overexpression defines a subpopulation of $ER^{+}/ERBB2^{-}$ breast cancer. Ectopic AT1R overexpression in normal breast epithelial cells conferred malignant invasion upon AT2 stimulation, which was attenuated by losartan, an AT1R antagonist normally used to treat hypertension [14]. These findings suggest an important role of AT1R activation in breast cancer and led us to explore whether an AT1R antagonist could suppress breast tumor growth by blocking angiogenesis *in vivo*.

2. Materials and methods

2.1. Cell lines, animals, and tissue specimens

Human breast cancer cell lines MCF-7, T47D, SKBR-3 and MDA-MB-231 were obtained from the Cancer Research Institute of Heilongjiang Province, China. Female BALB/c nude mice (6–8 weeks old) were purchased from Beijing Laboratory

Abbreviations: AT, angiotensin; AT1R, angiotensin II type 1 receptor; MVD, microvascular density; RT-PCR, reverse transcription-polymerase chain reaction; VEGF, vascular endothelial growth factor.

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Animal Center of the Chinese Academy of Sciences and maintained under specific pathogen-free conditions. All animal experiments were approved by Harbin Medical University. Samples from 102 patients with histopathologically confirmed breast cancer were included in this study. The patients were female (age range 25–70 years, with a median age of 45 years), hospitalized at the Affiliated Tumor Hospital of Harbin Medical University from 1999 to 2001 and followed-up until January 2012. Formalin-Fixed, Paraffin-Embedded (FFPE) tissues and complete clinical records of patients were collected. None of the patients had undergone preoperative chemotherapy or radiotherapy. The study was approved by the Ethics Committee of Harbin Medical University.

2.2. Reagents, drugs and antibodies

Angiotensin II was purchased from Sigma (USA). Losartan potassium tablets were purchased from Merck & Co., Inc. (USA). Candesartan cilexetil tablets were purchased from Takeda Pharmaceutical Co., Ltd. (Japan). Rabbit anti-human AT1R polyclonal antibody was purchased from Santa Cruz (USA). Rabbit anti-human CD34 polyclonal antibody and rabbit anti-mouse β-actin polyclonal antibody were purchased from Boster (Wuhan, China). Rabbit anti-human VEGF-A polyclonal antibody was purchased from Zhongshan Goldenbridge (Beijing, China).

2.3. Immunohistochemistry (IHC)

Each experiment had a positive control, which was provided with the IHC kit. The negative control consisted of experiments where the primary antibody was replaced with PBS. Staining intensity was categorized as strong (score 3), moderate (score 2), weak (score 1), or absent (score 0). Staining extent was scored using the scale of 1, 2 or 3 when positive staining was detected in <30%, 30–75%, or >75% of the cell population, respectively. The final immunoassay score was calculated by adding the staining intensity and staining extent scores. The scores of 0–4 were considered as low intensity and 5–6 as high intensity. Microvascular density (MVD) was assessed using a light microscope in a single area of tumor (200×) representative of the highest MVD (neovascular "hot spot") [15,16]. The brown-stained single cells or cell clusters are counted as a blood vessel if it was clearly separated from its surrounding capillaries, tumor tissue or connective tissue components regardless of lumen. The average of standard records of five horizons was defined as a score of MVD.

2.4. MTT assay

Breast cancer cells in logarithmic growth phase were digested with trypsin, harvested, adjusted to a density of 2×10^3 cells/mL and transferred to 96-well plates at a volume of 100 μL per well. After 24 h when cells formed a monolayer, drugs at different concentrations (angiotensin II at concentrations of 1, 10, 100, 1000 or 10000 nmol/L; and losartan potassium at concentrations of 0.01, 0.1, 1, 10 or 100 μmol/L) were added. After incubated for 24 h, MTT solution (5 mg/mL) was added (20 μL/well). Cells were incubated at 37 °C for 4 h. Then the culture supernatant was removed and DMSO was added (100 μL/well). Cells were incubated in a shaker at 37 °C for 10 min until crystals were completely dissolved. The absorbance at 490 nm was determined using a microplate reader. Each experiment was performed in quintuplicate.

2.5. RT-PCR

Cells were seeded in culture bottles at a density of 2×10^5 cells/mL and cultured in 3 mL of RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. Different concentrations of angiotensin II and losartan potassium were added. The experiments were performed in triplicate. Cells were cultured for 4 h and harvested to isolate total RNA. After measurement of RNA concentration by ultraviolet spectrophotometry and detection of RNA integrity by gel electrophoresis on a 1% agarose gel, reverse transcription was carried out as follows: incubation at 42 °C for 60 min, inactivation of reverse transcriptase at 70 °C for 15 min and cooling at 5 °C for 5 min. β-actin was used as an internal control. The primers that used to amplify the cDNA were as follows: F: 5′-CTACAATGAGCTGCGTGTGGC-3′, R: 5′-CAGGTCCAGACGCAGGATGGC-3′ for β-actin; F: 5′-ATCCCAGAAAGTCGGCACCAGATG-3′, R: 5′-TGACTTTGGCTAC AAGCATTGTGCG′ for AT1R; and F: 5′-AAGTGGTGAAGTTCATGGATG-3′, R: 5′-CTGCATGGTGATGTTGGAC-3'for VEGF-A.

2.6. Western blotting

Tissue samples were homogenized and total protein concentration in each sample was determined using the Bradford assay. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed at a constant voltage of 100 V for 1 h. After electrophoresis, proteins were electrically transferred onto a nitrocellulose membrane at a constant voltage of 45 V for 1 h. After washing three times (5 min each) in TBST buffer with shaking at room temperature, the membrane was incubated overnight with the blocking solution (1% bovine serum albumin) at room temperature. The membrane was then incubated with primary antibody (anti-VEGF-A antibody; 1:500 dilution) for 2 h at room temperature, followed by washing with TBST buffer three times (10 min each). Subsequently, the membrane was incubated with alkaline phosphatase-labeled goat anti-rabbit secondary antibody (1:500) at room temperature for 1 h, followed by washing with TBST three times (10 min each) and TBS twice (10 min each). BCIP/NBT substrate solution was used for color development to detect the expression of VEGF-A protein. Images were photographed using the Bio-Rad gel imaging system (Bio-Rad, USA).

2.7. Nude mouse tumor xenograft model

The breast cancer cells with the highest AT1R expression were adjusted to a density of 2.5×10^7 cells/mL using serum-free RPMI 1640 medium. Trypan blue staining indicated the ratio of viable cells was >95%. Tumor cells (5×10^6 in 0.2 mL of HBSS) were suspended in serum free-RPMI/Matrigel mixture (1:1 volume) and injected into the upper right and left mammary pads of the nude mice by using a 22-gauge needle. Strict aseptic technique was followed during the entire process. The mean volume of the implanted tumors at the time of inoculation was about 5 mm^3, without differences among various groups. A total of five mice per group were used. Three days after the injection and every fifth day thereafter, the length and width of the tumors that developed will be measured. Eight days after inoculation, drugs were given every day. Mice were sacrificed at 30 days and final tumor mass and volume were recorded. Tumor volume was calculated as (length/2) × (width^2). Tumors were harvested; half of each tumor was frozen in liquid nitrogen and stored at −80 °C; half was fixed in 4% paraformaldehyde and stored at 70% ethanol.

2.8. Statistical analysis

The chi-square test and *t*-test were used to examine the differences in categorical variables and numerical variables between the two groups, respectively. The MVD values were expressed as mean ± standard deviation. The statistical analyses were conducted using the SPSS12.0 software. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Expression of AT1R in breast cancer cells

AT1R was positively expressed in MCF-7, T47D, and SKBR-3 breast cancer cells. The AT1R mRNA level was highest in MCF-7 cells (Fig. 1).

3.2. Effects of angiotensin II and losartan on the proliferation of MCF-7 cells

The 1st group of MCF-7 cells treated with angiotensin II at concentrations of 100, 1000 or 10000 nmol/L had enhanced proliferation compared to control cells ($P < 0.0001$; Fig. 2A). The 2nd group of MCF-7 cells directly treated with losartan potassium exhibited proliferation similar to the control cells ($P = 0.528$; Fig. 2B). The 3rd group of MCF-7 cells were incubated with 100 nmol/L of angiotensin II for 4 h, and then treated with losartan potassium for 24 h. The cells treated with losartan potassium at concentrations of 10 or 100 μmol/L showed significantly decreased proliferation compared to control cells ($P < 0.0001$; Fig. 2C).

3.3. Effects of angiotensin II and losartan on the expression of VEGF-A mRNA in MCF-7 cells

The 1st group of MCF-7 cells was incubated with 100 nmol/L of angiotensin II for 12, 24 or 48 h, respectively. VEGF-A mRNA levels

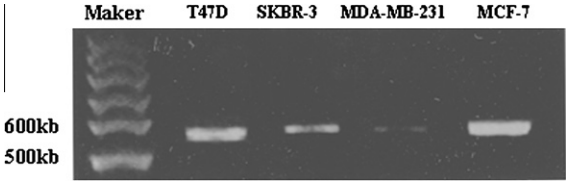

Fig. 1. Expression of AT1R in various breast cancer cell lines (400×).