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Angiotensin-(1-7) regulates angiotensin II-induced matrix metalloproteinase-8 in vascular smooth muscle cells

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ABSTRACT

Background and aims: Angiotensin II (Ang II) is a bioactive peptide that is related to cardiovascular disease such as atherosclerosis, whereas angiotensin-(1-7) (Ang-(1-7)) is a counter-regulator of angiotensin II, which protects against cardiovascular disease. Matrix metalloproteinase 8 (MMP-8) is thought to participate in plaque destabilization though degradation of extracellular matrix, improving the development of atherosclerosis. Whether Ang-(1-7) modulates Ang II-induced MMP-8 remains unclear. In this study, we investigated the effect of Ang-(1-7) on Ang II-induced MMP-8 expression in smooth muscle cells.

Methods: Smooth muscle cells were treated with Ang II, Ang-(1-7) and their antagonists. In addition, ApoE knockout mice were fed a high fat diet and subcutaneously injected with Ang II, Ang-(1-7), Ang II+Ang-(1-7) (±A779).

Results: We found that Ang II increased MMP-8 mRNA and protein expression in vascular smooth muscle cells, while Ang-(1-7) alone had no effect. However, Ang-(1-7) inhibited Ang II-induced MMP-8 expression. The inhibitory effect of Ang-(1-7) could be abolished by the competitive antagonist of Ang-(1-7) at the MAS receptor. Furthermore, Ang II induced p38 MAPK activation, and this was inhibited by the treatment of Ang-(1-7). Ang II-induced MMP-8 expression could be attenuated by the p38 MAPK inhibitor SB203580. Ang-(1-7) also significantly suppressed Ang II-induced MMP-8 in both atherosclerotic plaques and serum in *ApoE*$^{-/-}$ mice. The atherosclerotic plaques in mice treated with Ang-(1-7) and Ang II appeared to be more stable with more type I collagen contents than those in mice treated with Ang II.

Conclusions: Our results suggest that Ang-(1-7) plays an important role in protecting against atherosclerosis via counter-regulation of Ang II-induced MMP-8.

1. Introduction

It is well established that extracellular matrix (ECM) is involved in the development of atherosclerosis. ECM is the major constituent of atherosclerotic plaque, and a reduction in ECM can lead to the erosion of atherosclerotic plaques and consequent incidence of myocardial infarction. Matrix metalloproteinases (MMPs) are zinc-dependent proteases that can degrade ECM [1]. Among MMPs, MMP-8 has been found to play an important role in the progression of atherosclerosis in our previous study [2,3]. MMP-8 can cleave several ECM proteins, particularly type I collagen, which accounts for two-thirds of total collagen in atherosclerotic plaque, consequently resulting in unstable plaques that are prone to rupture [4]. It has been shown that MMP-8 increases the formation of atherosclerotic plaques in *ApoE*$^{-/-}$ mice, and MMP-8 concentrations are associated with the existence and severity of atherosclerosis and with cardiovascular outcome in patients [2,5–8]. One important source of MMPs is smooth muscle cells (SMCs), the major cellular components of the arterial wall. During the process of atherosclerosis, vascular SMCs migrate from media into intima, where they

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proliferate and produce MMPs [9]. Investigating the factors that regulate MMP-8 in vascular SMC may be helpful to find the strategy to prevent the development of atherosclerosis.

Angiontensin II (Ang II), one of the renin-angiotensin system (RAS) members, is well established as a bioactive peptide that regulates cardiovascular physiological responses, and overproduction of Ang II is related to cardiovascular disease such as atherosclerosis [10]. Activation of Ang II AT1 receptor leads to pro-inflammation, proliferation, vasoconstriction, and ECM remodeling [11]. Previous studies have shown that Ang II can stimulate SMCs to produce MMPs, including MMP-1, MMP-2, MMP-3 and MMP-9 [12–14]. However, little is known about the effect of Ang II on MMP-8 expression, the key enzyme for degradation of type I collagen in atherosclerotic plaque.

Angiotensin-(1-7) (Ang-(1-7)), another bioactive peptide of RAS, has been shown to counter-regulate many actions of Ang II. Angiotensin-converting enzyme 2 (ACE2) can convert the octapeptide Ang II to the heptapeptide Ang-(1-7). Ang-(1-7) shows protection against cardiovascular diseases, for example, the suppression of the atherosclerotic plaque progression [15]. Activation of Ang-(1-7) MAS receptor results in vasodilation, anti-proliferation and anti-inflammation activities [16]. Therefore, Ang-(1-7) antagonizes myocardial hypertrophy during hypertension [17], and attenuates the constriction of blood vessels induced by Ang II [18]. Nevertheless, whether Ang-(1-7) can regulate MMP-8 expression induced by Ang II has not been investigated.

Therefore, this study aims to clarify the role of Ang-(1-7) in atherosclerosis with respect to two aspects: (1) whether Ang-(1-7) can modulate Ang II-induced MMP-8 expression in SMCs; and (2) whether Ang-(1-7) can influence Ang II-induced MMP-8 expression in atherosclerotic plaque *in vivo*.

2. Materials and methods

2.1. Cell culture and treating conditions

Human primary aortic SMCs were purchased from ScienCell (Carlsbad, CA) and cultured in DMEM with 10% FBS. Cells from passages 4-8 were used after serum depletion for 24 h prior to treatment. Treating conditions were as follows: 0.1 μmol/L Ang II, 0.1 μmol/L Ang-(1-7), 1 μmol/L losartan and 1 μmol/L A779. Ang II was added 10 min after Ang-(1-7). To study whether Ang II and Ang-(1-7) mediate their effects through their specific receptors, SMCs were first treated with antagonist of AT1 receptor, losartan, or antagonist of MAS receptor, A779, for 5 min, and then further treated with Ang II or Ang-(1-7). DMEM and FBS were from Invitrogen. Ang-(1-7) and Ang II were purchased from Sigma Aldrich.

2.2. Experimental animals

Eight week old *ApoE*$^{-/-}$ mice were fed a high fat diet (0.15% cholesterol and 21% fat) for 8 weeks to construct the atherosclerotic model. After 4 weeks of high fat diet, mice were divided into 5 groups (n = 10 in each group) with a chronic infusion of saline, Ang II (1000 ng kg^{-1}.min^{-1}), Ang-(1-7) (400 ng kg^{-1}.min^{-1}), Ang II (1000 ng kg^{-1}.min^{-1})+Ang-(1-7) (400 ng kg^{-1}.min^{-1}), and Ang II (1000 ng kg^{-1}.min^{-1})+Ang-(1-7) (400 ng kg^{-1}.min^{-1})+A779 (400 ng kg^{-1}.min^{-1}) for 4 weeks, respectively. All mice received the 4-week subcutaneous injection using subcutaneous osmotic pumps (Alzet, Alza Corp., USA). After 8 weeks of high fat diet, mice were sacrificed and tissues were collected for analysis.

All animal procedures were conducted according to the Animal Management Rule of the Chinese Ministry of Health (documentation 55, 2001) and approved by the Peking University People's Hospital Animal Care and Use Committee.

2.3. RT-PCR analysis

SMCs were treated with Ang II, or Ang-(1-7), or together with their antagonists. Six hours later, total RNA was extracted and cDNA was synthesized with the SuperScript First-strand Synthesis System (Invitrogen). The expression of *MMP-8* mRNA was measured by SYBR Green I fluorescence method and detected in Bio-Rad Mini-Opticon. The primer sequences of *MMP-8* were 5′-GTGGGAACG-CACTAACTTGACC-3' (forward) and 5′-GAAGATGAGAGGTGATGCAACACT-3' (reverse). In addition, *TIMP-1, TIMP-2, TIMP-3* and *TIMP-4* levels were measured with real-time RT-PCR as well. Primer sequences are shown in Supplementary Table 1. The results were calculated by the method of delta-delta C_T, using β-actin as standardization.

2.4. Western blotting

SMCs were incubated with or without losartan or A779 for 5 min, and then further treated with Ang II, Ang-(1-7), separately or together for 16 h. Cell lysates were prepared in RIPA lyses buffer (50 mM Tris HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1.2% Triton-X-114, 1 mM NaF, 200 mM $NaVO_4$) with protease inhibitor (Roche Diagnostics). Protein concentrations were determined by the BCA protein assay method. Equal amounts of protein extracts were loaded into 10% SDS-polyacrylamide gels and then transferred to PVDF membranes. The membranes were incubated in 5% milk in Tris-buffered saline Tween 20 to block the non-specific binding. The membranes were incubated with primary antibodies (MMP-8, t-p38 MAPK, p-p38 MAPK, ERK1/2, *p*-ERK1/2, β-actin) overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The membrane was detected with an enhanced ECL chemiluminescence system (Millipore). Quantity One 4.6.2 software was used to measure the densitometric signal intensity. Immunoblot analysis of β-actin was conducted to ensure equal protein loading.

2.5. Atherosclerotic lesion analysis and immunohistochemical staining

The mouse aortic roots were separated and fixed in 4% paraformaldehyde. Paraffin-embedded sections were cut serially at 5 μm intervals from the aortic sinus. For area measurements, the sections were stained with hematoxylin and eosin. Total lesion area was quantified by averaging 6 sections that were spaced 30 μm apart, starting from the base of the aortic root. Images were captured by Microscope and analyzed using ImageJ Software. The aorta was also fixed in 4% paraformaldehyde and embedded in paraffin block. The sections were cut at 5 μm thickness and stained with hematoxylin and eosin. Elastin was stained using elastin van Gieson method. Immunofluorescence of aortic roots was performed using primary antibodies for MMP-8 (Santa Cruz Biotech.), SMC marker α-actin (Sigma Aldrich), endothelial cell marker CD31 (Abcam) and macrophage marker CD68 (Abcam). In addition, the paraffin sections of aortic roots were subjected to immunostaining with antibodies for MMP-8 (Santa Cruz Biotech.) and type I collagen (Abcam). In brief, the paraffin embedded sections were deparaffinized with xylene and ethanol, followed by antigen retrieval in 10 mM citrate buffer (pH 6.0) for 20 min. The endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide for 15 min. To block nonspecific binding, sections were incubated with 5% animal serum for 20 min. Sections were then incubated with primary antibodies overnight at 4 °C. The slides were washed with PBS and incubated with the secondary antibody for 30 min. The color was developed using 0.03% hydrogen peroxide

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