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Angiotensin-II and vascular endothelial growth factor interaction plays an important role in rat liver fibrosis development

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Abstract

Both angiotensin-II (AT-II) and vascular endothelial growth factor (VEGF) have been shown to play important roles in the progression of liver fibrosis. However, the interaction of AT-II with VEGF in the liver fibrosis has not been elucidated yet. The aim of the current study was to elucidate a possible association between these molecules, especially in conjunction with the hepatic stellate cells (HSC). The effect of AT-II type 1 receptor blocker (ARB) was assessed on several indices of choline-deficient L-amino acid-defined (CDAA)-induced liver fibrogenesis. This ARB significantly suppressed liver fibrosis development along with suppression of the VEGF expression and neovascularization in the liver. In the cultured activated HSC, AT-II induced VEGF in a dose- and time-dependent manner. ARB and LY333531, a protein kinase C (PKC) inhibitor, attenuated this augmentation. These results indicated that AT-II and VEGF interaction played an important role in liver fibrosis development, and that in the activated HSC, AT-II utilized type 1 receptor and PKC as an intracellular signaling pathway to induce VEGF.

Keywords: Angiogenesis; Angiotensin-II; Hepatic stellate cell; Liver fibrosis; VEGF

1. Introduction

It is now recognized that several cytokines, which interact with each other, play pivotal roles in the progression of liver fibrosis development [1]. Among them, both angiotensin-II (AT-II) and vascular endothelial growth factor (VEGF) have been shown to be key modulators in liver fibrosis development [2,3]. AT-II, which is an octapeptide produced mainly by the proteolytic cleavage of its precursor angiotensin-I by angiotensin-I converting enzyme (ACE), has also been shown to induce contraction and proliferation of the activated hepatic stellate cells (HSC), which play a pivotal role in liver fibrosis development [4]. It has been reported that inhibition of the rennin–angiotensin system (RAS) by ACE-inhibitor (ACE-I) and AT-II type 1 receptor (AT1-R) blocker (ARB) significantly suppressed liver fibrosis development in several animal experimental models along with suppression of HSC activation [2,3,5]. Furthermore, recent studies have revealed the clinical usefulness of ARB in prevention of liver fibrosis progression in several types of chronic liver diseases [6,7].

Emerging evidences have shown that angiogenesis plays a pivotal role in many physiological and pathological processes, such as tumor growth, arthritis, psoriasis, and diabetic retinopathy [8,9]. Although previous studies conducted to determine the molecular process associated with fibrosis and angiogenesis were performed independently, recent studies have revealed that both biological phenomena emerged synergistically [10]. It was shown that neovascularization signif-

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icantly increased during the development of liver fibrosis in human and animal experimental studies [11–13]. Angiogenesis is regulated by the net balance between the pro-angiogenic factors and angiogenic inhibitors. Among the identified pro-angiogenic factors, the vascular endothelial growth factor is the most potent factor in the angiogenesis process [14]. It has been reported that the VEGF expression significantly increased during the liver fibrosis development, and suppression of VEGF signaling cascade markedly attenuated liver fibrogenesis along with inhibition of HSC activation [2]. The VEGF gene expression is induced by several types of cytokine, and recent studies have shown that AT-II also induces the VEGF expression in several types of cells [15]. However, the interaction between AT-II and VEGF in the HSC has not been elucidated yet.

Protein kinase C (PKC) is composed of a family of serine–thereonine kinases. It has been postulated that activation of PKC is an important intracellular signaling pathway for several biological properties, such as cellular differentiation or growth [16]. It has been shown that AT-II stimulates production of tissue inhibitor of metalloproteinases-1 (TIMP-1) via a PKC-dependent pathway in the activated HSC *in vitro* [17]. VEGF also has been shown to utilize PKC as an intracellular signaling in the endothelial cells (EC) [14].

In the present study, we examined the interaction between AT-II and VEGF in rat liver fibrosis development. Moreover, we also examined the interaction between these molecules in the activated HSC with a special focus on the PKC signaling cascade.

2. Methods

2.1. Animals and compounds

A total of 40 male Fisher 344 rats, aged 6 weeks, were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). They were housed in stainless-steel, mesh cages under control conditions of temperature ($23 \pm 3\,^{\circ}C$) and relative humidity ($50 \pm 20\%$), with 10–15 air changes per hour and light illumination for 12 h a day. The animals were allowed access to food and tap water *ad libitum* throughout the acclimatization and experimental periods. Candesartan (CA; an ARB) and LY333531 (a PKC specific inhibitor) were supplied by Takeda Pharmaceutical Co. (Osaka, Japan) and Lilly Laboratories (Indianapolis, IN, USA), respectively. The choline-deficient L-amino acid-defined (CDAA) diet and its control, a choline-supplemented, L-amino acid defined (CSAA) diet, with the previously described composition [18], were obtained from Dyets Inc. (Bethlehem, PA, USA).

The experimental period in all experiments was 16 weeks. The rats in Groups 1 and 2 (G1 and G2) received the CDAA diet, and the CSAA diet was given to the rats in G3 and G4 ($n = 10$ in each group). Then, 2 mg/kg/day of CA were administered to G1 and G3 by gavage. At the end of all experiments, all rats were killed under ether anesthesia, and examined for the study items. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals.

2.2. Histopathological examinations and several markers

Five-micrometer-thick sections of formalin-fixed and paraffin-embedded livers were processed routinely for Sirius-red (S-R) staining for detection of liver fibrosis development. Immunohistochemical staining of α-smooth muscle actin (α-SMA) (DAKO, Kyoto, Japan) was performed as described previously [3]. Computer-assisted semi-quantitative analyses of fibrosis development and α-SMA-immunopositive cells were carried as described previously [3]. Regarding the α-SMA-positive cells, we only examined the sinusoidal lining for image analysis.

2.3. Culture of HSC

The HSC were isolated from the liver of F344 rats as described previously [17]. The cells were plated at a density of 5×10^5 cells/ml on uncoated 60-mm plastic dishes. After 5 days in culture, HSC became myofibroblast-like with reduced lipid vesicles and increased immunoreactive α-SMA, and 7 days after plating, all of the cells were well-spread and α-SMA positive. On day 10, the activated HSC were arrested by serum-starvation for 24 h to avoid interference with the serum components. AT-II (10^{-10} to 10^{-6} M) (Nacalai, Kyoto, Japan), CA (10^{-6} M), and LY333531 (10^{-6} M) were added for the respective studies.

2.4. Analysis of the mRNA expressions of CD31 and VEGF by real-time PCR

The mRNA expressions of CD31, which is widely used as a marker of neovascularization, and VEGF were evaluated by real-time PCR as described previously [19]. For cDNA synthesis, Taqman reverse transcription reagents with oligo-dT primer were used as described in the manufacturer's manual of the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA), which was used for real-time PCR amplification following the Taqman Universal PCR Master Mix Protocol (PE Applied Biosystems). Relative quantification of the gene expression was performed as described in the manual using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The threshold cycle and the standard curve method were used for calculating the relative amount of target RNA as described for PE. The following temperature regimen was employed: holding at 50 °C for 2 min, holding at 60 °C for 30 min, holding at 94 °C for 5 min, cycle 45 repeats at 94 °C for 1 min, at 55 °C for 1 min, and at 72 °C for 1 min. To prevent genomic DNA contamination, all RNA samples were