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Original Article

Serum amyloid P component regulated the development of diabetic nephropathy via down regulation of CCL-1

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Abstract: Serum amyloid P component (SAP) is a plasma protein which has calcium-dependent binding activity and belongs to the pentameric protein family. It is mainly regulated in the inflammation in mice. Diabetic nephropathy is a common chronic disease. Studies show that SAP is associated with amyloidosis, Alzheimer's disease, apoptosis, fibrosis, inflammation, and other autoimmune diseases. The role of SAP in diabetic nephropathy has not yet been reported. Therefore, this study intends to use SAP transgenic (SAP-Tg) mice to investigate the role of ASP in diabetic nephropathy. SAP-Tg mice and C57 mice were included to establish models of diabetic nephropathy. Protein microarray technology was used to select differentially expressed proteins in serum of both SAP-Tg mice and C57 mice. ELISA and real time PCR were used to confirm the expression level of selected proteins. Compared to C57 mice, SAP-Tg mice with diabetic nephropathy showed less morphological changes. Protein microarray screening results show that chemokine C-C motif ligand 1 (CCL-1) was differentially expressed. Real time PCR results showed that CCL-1 of SAP-tg mice was significantly lower than that of C57 mice at mRNA level. SAP recombinant proteins was used stimulate RAW cells (mouse macrophage cell line) and ELISA results showed that CCL-1 levels were significantly lower than that of the control group. Peritoneal macrophages of both SAP-Tg mice and C57 mice were isolated and culture. The supernatant Primary cell culture supernatant ELISA results showed supernatant of peritoneal macrophages from SAP-Tg mice has less levels of CCL-1 than that of C57 mice. SAP inhibited the development of diabetic nephropathy by reducing the secretion of CCL-1 from macrophages.

Keywords: SAP, diabetic nephropathy, CCL-1

Introduction

The development of kidney disease is a complex process involving a series of physiological changes, including cell proliferation, apoptosis, cell invasion, and metastasis. Clinical symptoms of renal cell carcinoma include anorexia, body weight loss, and so on. Serum amyloid P component (SAP) belongs to the pentameric protein family (pentraxins). Synthesized by liver, SAP is a plasma protein which has calcium-dependent binding activity. SAP and CRP are dramatically upregulated in acute inflammation of mice and human being. CRP and SAP are regulated by a series of cytokines (such as IL-1, IL-6, and TNF-α, etc.). Under physiological state, SAP is composed of the five identical subunits. SAP is an important component of normal human kidney tissue structure. Studies have shown that SAP can bind extracellular amyloid to resist the decomposition by protease and maintain in vivo amyloid deposits [1]. It is thought that SAP is related to Alzheimer's disease, type I diabetes mellitus, and amyloidosis.

Chemokines play an important role in cancer development. Chemokine receptors are mainly expressed on the surface of white blood cells, endothelial cells, skin cells, nerve cells, and certain types of tumor cells. CCL-1 is a chemokine that found to have a significantly physiological significance which is mainly secreted by immune cells and macrophages. CCR8 is the only known receptor of CCL-1. Studies have shown that CCL-1 can affect nerve pain be regulating of glia, regulate the migration of nerve cells [2], promote the normal development of

the thymus, and play an important role in allergic reactions and asthma. It has been shown that levels of CCR8 were increased in patients with severe diabetic nephropathy [3].

In order to study the relationship between SAP and diabetic nephropathy, we constructed SAP-Tg mice. Briefly, cDNA of mouse SAP injected into fertilized eggs and transferred to surrogate mice to obtain chimeric mice (SAP-Tg +/-). Mating of chimeric mice (SAP-Tg +/-) to get SAP-Tg mice were then backcrossed with C57 mice for at least eight generations to get mice with C57 background with overexpression of mSAP. In transgenic mice as a tool rat, biological phenotype is stable, reproducible results and so on. SAP-Tg mice were used to establish mouse model of diabetic nephropathy and results showed that SAP can inhibit the rapid development of diabetic nephropathy by reducing secretion of CCL-1 by macrophages.

Materials and methods

Establishment of mouse model of diabetic nephropathy

SAP-Tg mice and C57 mice were purchased from Guizhou Medical University. All mice were fasted for 8-12 h. Streptozotocin (STZ) was injected intraperitoneally with a dose of 45-65 mg/kg body weight. Blood samples were collected 48-72 h after injection from tail vein to measure the concentration of blood sugar. Concentration of blood sugar greater than 16.7 mmol/L, and urine glucose at 3+ -4+ for more than one week means the establishment of diabetic nephropathy model [4]. A total of 48 mice with diabetic nephropathy were divided into experimental group (24 SAP-Tg mice) and control group (34 C57BL/6J mice) for later study.

Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Guizhou Medical University.

Real-time PCR

Fresh tissue was pulverized in a mortar with liquid nitrogen, mixed with 1 ml Trizol solution, incubated at room temperature for about 10 min, mixed violently with 0.2 ml chloroform for 15 seconds, and incubated at room temperature for 2-3 min, centrifuged at 12000 g for 15 min at 4°C. The supernatant (0.5 ml) was transferred into a new centrifuge, mixed with 0.5 ml isopropanol, incubated at room temperature for 10 min, centrifuged at 12000 g for 10 min at 4°C. The supernatant was discarded. The pellet was washed with 1 ml 75% ethanol and re-suspended in 20 μl DEPC water. RNA was quantified and reverse transcribed into cDNA for PCR. Primers were synthesized by Shanghai Biological Co with following sequences: CCL-1: F-AGCTTCAGGCAGGCAGTATC; R-CATCTCGGAGCCTGTAGTGC; GAPDH: F-GCCCTGAGGGCCCGAACTGTTACT; R-CAGACGCACGGCTTTGACCTTCTT.

Protein array

BCA colorimetric kit was used for protein quantification. Protein concentration was adjusted to 500 μg/ml. 200 μl of sample was loaded to protein microarray (Affymetrix USA) and incubated at room temperature in the dark. After 3 times washing, primary antibody (provided in the kit, 1:100 dilution) was added and incubated overnight. After 3 times washing, secondary antibody (provided in the kit, anti-goat, 1:100 dilution) was added and incubated at room temperature for 1 h. Membranes were then washed and developed.

ELISA

ELISA kit was purchased from Guangzhou Rui Boao Biotechnology Company. Proteins were extracted. 100 μl of protein samples were added to each well and incubated at 37°C for 90 min. 100 μl of biotin-labeled antibody was added to each well and incubated at 37°C for 60 min. Plates were washed with 0.01 M TBS for 3 times. Added per well 100 μl of ABC was added to each well and incubated at 37°C for 30 min. TMB was added to each well and incubated at 37°C in the dark for 30 min to stop the reaction. Absorbance at 470 nm was recorded using a microplate reader.

Statistical analysis

SPSS11.0 was used for statistical analysis. Data was analyzed using t-test, Pearson correlation analysis, or χ^2 test. $P<0.05$ was considered statistically significant.

Results

Protein microarray screening of candidates of SAP

H-E staining results showed that SAP-Tg mice had less pathological damage compared with

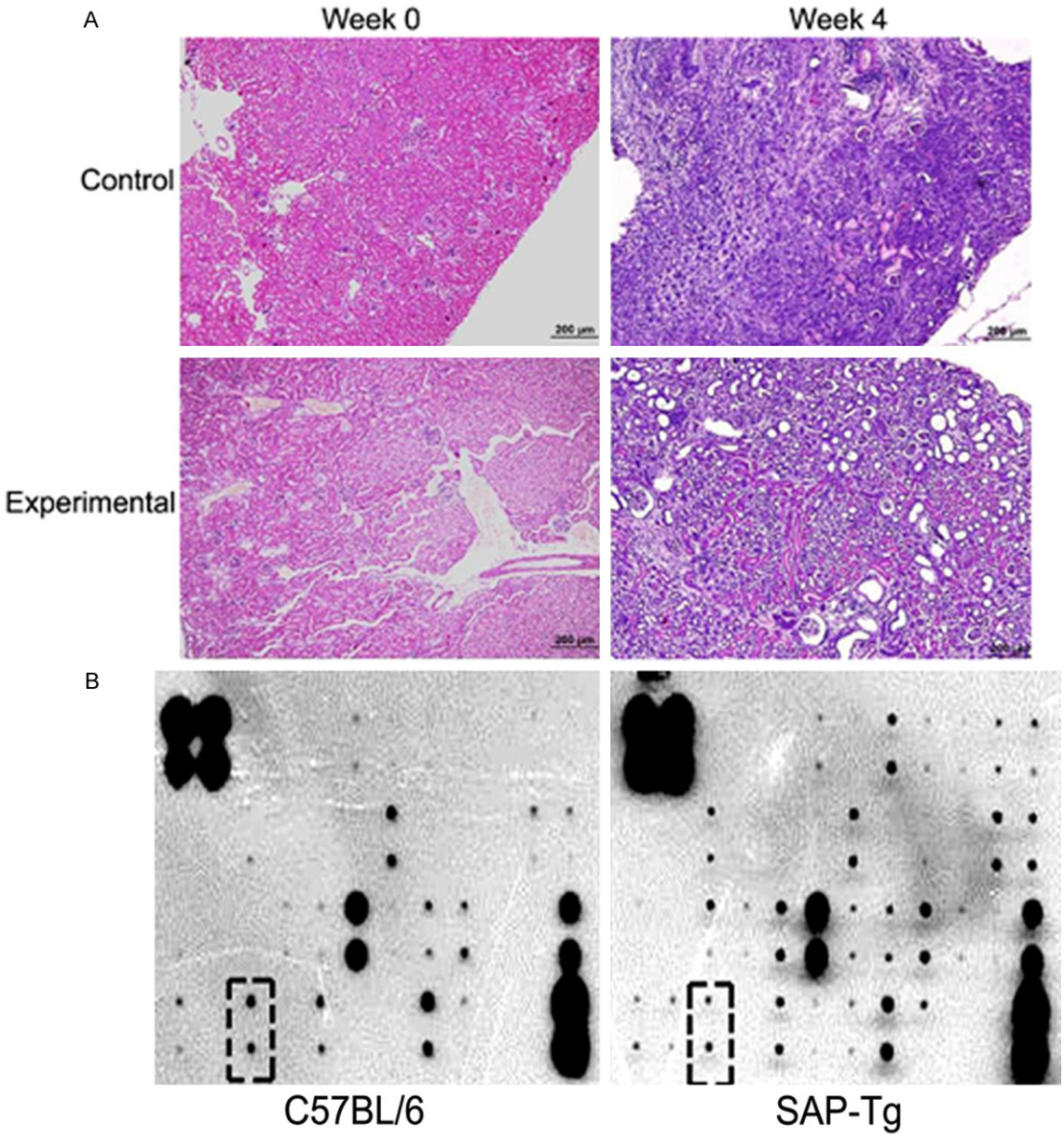

Figure 1. Protein microarray screening of candidate factors of SAP. A. H-E staining. B. Protein microarray screening of cytokines.

normal control mice (**Figure 1A**). Protein microarray results (**Figure 1B**) showed that SAP-Tg mice had lower levels of CCL-1 compared with control mice.

Differential expression of CCL-1 in SAP-Tg mice and C57 mice

In order to know whether there are differences in the expression levels of CCL-1 at mRNA level in both SAP-Tg mice and C57 mice, total RNA was extracted and real-time PCR was used after reverse transcription. Results (**Figure 2**) showed that the expression level of CCL-1 of SAP-Tg mice was significantly lower than that of C57 control mice. ELISA results showed that the protein level of CCL-1 of SAP-Tg mice was significantly lower than that of C57 control mice.

Effect of SAP on the secretion of CCL-1

ELISA was used to measure the effect of SAP protein (50 ng/ml) on the secretion of CCL-1

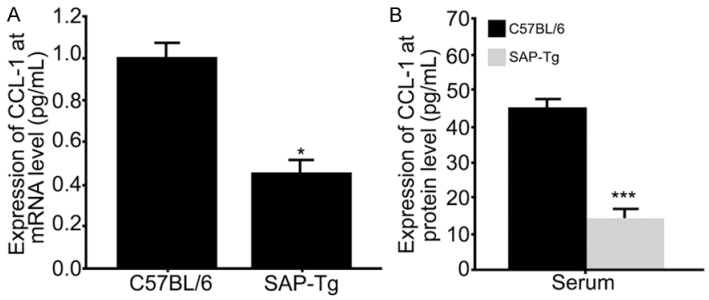

Figure 2. Differential expression of CCL-1 in SAP-Tg mice and C57 mice. A. CCL-1 of SAP-Tg mice and C57 mice at mRNA level; B. CCL-1 of SAP-Tg mice and C57 mice at protien level. *P<0.05, compared with control; ***P<0.001, compared with control.

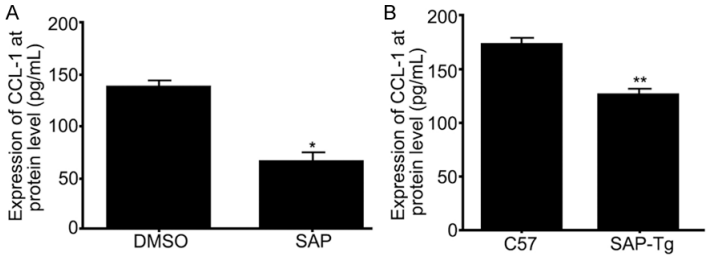

Figure 3. Effect of SAP on the secretion of CCL-1. A. SAP suppressed the secretion of CCL-1 by murine macrophage RAW264.7 cells. B. Macrophages from SAP-Tg mice secreted less CCL-1. *P<0.05, compared with control; ***P<0.001, compared with control.

using RAW264.7 cells. Results (**Figure 3**) showed that SAP treatment significantly lower the level of CCL-1 secreted by RAW264.7 cells.

In order to detect the effects of the effect of SAP on the secretion of CCL-1 in SAP-Tg mice, LPS was intraperitoneally injected to activate the macrophages. ELISA results (**Figure 3**) showed that macrophages from SAP-Tg mice secreted less CCL-1 than that of C57 mice.

Discussion

SAP is a serum protein produced by the liver which increases dramatically in acute inflammation in mice, but not in human. Current study on SAP is focused on amyloidosis, AD-like symptoms, autoimmune, bacterial infection, vascular disease and fibrosis. Darrell Pilling found APCS-/- mice were protected from lung injury and pulmonary fibrosis compared with C57 mice [5]. Studies have shown that deletion of SAP alleviated pathological changes of atherosclerosis in APOE-/- mice [6]. However, the role of SAP in diabetic nephropathy has not been reported.

To further explore the role of SAP in diabetic nephropathy, we successfully constructed diabetic nephropathy model in both SAP-Tg mice and C57 mice. H-E staining showed that kidneys of SAP-Tg mice were less damaged than that of C57 mice. Protein micrroarray screening showed that the expression levels of CCL-1 in SAP-Tg mice were significantly lower than that of C57 mice. It is known that chemokines play an important role in cancer development. The receptors of chemokines were expressed mainly on the surface of white blood cells. Studies have shown that chemokines can activate their receptors which in turn cause the movement of leukocytes [7]. But whether the chemokines have chemotactic effect to other types of cells, particularly tumor cells, is still unknown. CCL-1 mainly secreted by activated T cells, mast cells, and macrophages secrete, is the first found chemokine in the CC family [8-12]. CCR8 is the only known receptor of CCL-1 [13, 14] which is mainly expressed in the lymphoid tissues [15-17]. The role of CCL-1 in the development of kidney is unclear. Previous studies showed that: CCL-1 has anti-apoptotic role in lymphoma and leukemia in adults [18-20]. This study showed that CCL-1 was differentially expressed in SAP-Tg mice and C57 mice at both mRNA level and protein level showed by real time PCR and ELISA. Although we verified that SAP suppressed the secretion of CCL-1 by macrophages using ELISA, we cannot rule out the effect of CCL-1 secreted by activated T cells and mast cells. How to exclude the effect of CCL-1 secreted by activated T cells and mast cells will be the key of our future study.

In conclusion, SAP suppresses the progression of diabetic nephropathy by reducing the secretion of CCL-1 by macrophages.

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Disclosure of conflict of interest

None.

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