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Increased cytoprotective function in the liver of transgenic mice expressing osteopontin in hepatocytes

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Abstract

Osteopontin is a crucial factor for initiation of Th1 immune reaction. Previously, we established transgenic mice expressing osteopontin in hepatocytes, in which lymphocyte infiltration occurred spontaneously at 12 weeks of age and liver necrosis at 24 weeks of age. This liver necrosis may develop through provocation by excessive Th1 immune reaction, but it is also possible that hepatocytes become fragile under abundant osteopontin in the cytoplasm. Thus, gene expression profiles in the liver were evaluated to seek such contributing factors in the transgenic mice. On DNA microarray analysis of 3774 mouse genes, 16 genes were selected as hepatic genes significantly up-regulated in the transgenic mice aged 8 weeks than in the negative littermate, which included mRNAs of cytoprotective metallothionein and glutathione *S*-transferase (GST). Hepatic up-regulations of both genes were also seen by Western blotting. Liver necrosis in the centrilobular areas developed after carbon tetrachloride treatment, but its histological extent and plasma ALT activities were significantly smaller in the transgenic mice aged 8 weeks than in the wild-type C57BL/6 control mice. We conclude that cytoprotective function of the liver is increased through up-regulated expressions of metallothionein and GST, and thereby susceptibility of hepatocytes to the stress may be less possible, if any, in the development of spontaneous liver necrosis in transgenic mice expressing osteopontin in hepatocytes.
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1. Introduction

Chronic hepatitis develops as a result of Th1 immune reaction in the liver provoked by hepatitis virus infection and autoimmunity against hepatocytes. Cytotoxic T lymphocytes (CTLs) activated by Th1 cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α are responsible for the initiation of hepatocyte necrosis and apoptosis [1,2], since they recognize virus-related antigens on hepatocytes and damage these cells via the perforin/granzyme B system [3]. Such damage is further intensified through the Fas/Fas ligand system and TNF-α [3]. The similar mechanisms might be involved in the development of autoimmune hepatitis, because CTLs can recognize auto-antigens expressed on hepatocytes.

Osteopontin is a secreted glycoprotein that can bind to hydroxyapatite and calcium [4]. This protein is expressed mainly in the bone and kidney [4,5], and is shown to contribute physiologically to extracellular matrix formation and calcium deposition in these organs [5]. Also, osteopontin can bind to αvβ3 integrin on monocytes and macrophages through the RGD motif [6], and act as a chemokine for these cells [6,7]. Moreover, osteopontin is a cytokine crucial for the initiation of Th1 immune reaction in mice [8]. Previously, we demonstrated that osteopontin expression was up-regulated in Kupffer cells, macrophages and stellate cells activating

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in the liver of rats after treatment with carbon tetrachloride (CCl_4) or *Propionibacterium acnes* [9,10] and of patients with fulminant hepatic failure [11], suggesting that this factor might be involved in the initiation of Th1 immune reaction in the liver under pathological conditions.

Recently, we established four lines of transgenic mice expressing osteopontin exclusively in hepatocytes using a vector containing human serum amyloid P component (SAP) promoter [12]. In these mice, mononuclear cell infiltration occurred in the liver later than 12 weeks of age, and focal liver necrosis developed with elevated serum ALT activities later than 24 weeks of age [12]. Also, antinuclear antibody was positive in the plasma in 50% of these transgenic mice, suggesting that the mice may be useful as a model of autoimmune hepatitis [12]. Activated CTLs might produce hepatocyte injury in the transgenic mice, because infiltrating cells were positive for CD8 and MHC class II on immunohistochemistry [12]. However, there remains a possibility that hepatocytes become fragile leading to development of liver necrosis spontaneously in these mice, since the expression profiles of various proteins including those responsible for cytoprotection might be modified in hepatocytes.

In the present paper, the expression profiles of various mRNAs were evaluated comprehensively by DNA microarray analysis in the liver of the transgenic mice, and cytoprotective function of hepatocytes was assessed when liver injury was provoked by CCl_4 administration.

2. Materials and methods

2.1. Osteopontin transgenic mice

The offspring of the transgenic mouse SAP-osteopontin 17 [12] were maintained on a commercial pelleted diet and water ad libitum in a room at $22 \pm 2\,^{\circ}C$ under normal laboratory lighting conditions. The male and female transgenic mice were mated each other at 8 weeks of age, and genomic DNA extracted from the tail of newborn mice was subjected to polymerase chain reaction (PCR) assay to detect the transgene. Sense and antisense primers were synthesized based on cDNA sequences of 5′ non-coding region of rabbit β-globin gene and the osteopontin gene, respectively; 5′-TGC TGT CTC ATC ATT TTG GC-3′ for sense primer and 5′-GCA GGC TGT ATA GCT TCT CCT-3′ for antisense primer [12]. Mice carrying the transgene at 8 weeks of age were subjected to the experiments. Also, negative littermates and C57BL/6 mice (wild-type of the transgenic mice) (Japan SLC, Hamamatsu, Japan) at 8 weeks of age were used for the experiments as controls of the transgenic mice. All animal protocols conformed to the Guide for Care and Use of Laboratory Animals by the National Academy of Sciences.

2.2. DNA microarray analysis

Broad scale gene expression was evaluated by Atlas Glass Mouse 3.8 Microarrays (Clontech Laboratories, Palo Alto, CA), which include 3774 mouse cDNA fragments. Total RNA was extracted from the liver of both transgenic mice and negative littermates according to the method by Chomczynski and Sacchi [13]. cDNA was synthesized from 20 μg of total RNA by conventional reverse transcription procedure and labeled by Cy3 fluorescent dye using Atras Glass Fluorecent Labeling Kit (Clontech Laboratories) [14]. Hybridization of microarrays was carried out in a hybridization solution (supplied by Clontech Laboratories) for 16 h at 50 °C. The microarray slides were washed according to the manufacture's protocols. Then, the slides were air-blown dried, and scanned with Gene Pix 4000B (Inter Medical Co. Ltd. Nagoya, Japan).

2.3. Western blot analysis

Excised livers from the transgenic mice, negative littermates and C57BL/6 mice were homogenized at 4 °C in a solution of 1 mM EDTA, 100 mM KCl, 1% Triton X-100 and 20 mM 2-amino-2-hydroxymethyl-1,3-propanediol-HCl (pH 7.4) with complete protease inhibitor cocktail. The resultant suspension was boiled for 5 minutes and disrupted by sonication. The samples containing 30 μg of protein were subjected to electrophoresis in SDS-polyacrylamide gel (12% acrylamide). Proteins in the gel were transferred onto nitrocellulose paper (Hybond-C; Amersham International plc, Buckinghamshire, England) using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Richmond, CA). The paper was soaked in a blocking agent made from skim milk (BLOCK ACE; Snow Brand Milk Product Co. Ltd., Sapporo, Japan), and exposed to polyclonal antibodies against recombinant human metallothionein (FL-6I; Santa Cruz Biothechnology Inc., Santa Cruz, CA) and purified human glutathione *S*-transferase (GST)-π (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) as primary antibodies and alkaline phosphatase-conjugated goat antibody against rabbit IgG (Immunotech, Marseille, France) as a secondary antibody. Products of the reaction were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT: GIBCO Laboratories) as a developing agent.

2.4. Liver injury in mice treated with CCl_4

Nine female transgenic mice and 11 female C57BL/6 mice received an intraperitoneal injection of CCl_4 as 20% solution in olive oil at 5.0 mL/kg body weight. They were sacrificed at 24 h later under anesthesia with ether, and blood was collected through the inferior caval vein using a syringe containing a 1:10 volume of 3.8% sodium citrate for preparation of plasma, followed by excision of the liver for histological examination. Three female transgenic mice were similarly sacrificed without treatment of CCl_4.

Plasma ALT activity was determined using a commercial kit (Iatron Laboratories Inc., Tokyo, Japan). Excised livers were fixed in 20% formalin neutral buffer solution (Muto Pure Chemicals Ltd., Tokyo, Japan) and embedded in paraf-

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