Possible Changes in Expression of Chemotaxin LECT2 mRNA in Mouse Liver after Concanavalin A-Induced Hepatic Injury

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The functions of leukocyte-derived chemotaxin 2 (LECT2), a novel liver-specific protein, are not well defined, especially after hepatic injury. The changes in expression of LECT2 mRNA were investigated after concanavalin A (Con A)-induced hepatic injury in mice. Serum glutamate pyruvate transaminase (s-GPT) activity and the percentage of liver DNA fragmentation, an indicator of hepatic apoptosis, increased 8 h after intravenous administration of Con A (13 mg/kg). Expression of LECT2 mRNA was reduced from 8–24 h after injection of Con A, but was detected again 48 h after recovery from hepatic injury. Expression of tumor necrosis factor (TNF)- α and interferon (IFN)- γ mRNA was observed in liver 2 h after Con A injection. Z-Val-Ala-Asp(OMe)-CH₂F (Z-VAD-FMK), a caspase inhibitor, was administered to mice to investigate whether LECT2 was involved in apoptosis of liver cells after Con A injection. Z-VAD-FMK inhibited s-GPT activity and DNA fragmentation in the liver 8 h after Con A-induced hepatic injury, but did not prevent the reduction of LECT2 mRNA, or induction of TNF- α and IFN- γ mRNA expression. When the relation between expression of LECT2, TNF- α and IFN- γ mRNAs was examined 8 h after Con A injection in wild-type or immunodeficient (nu⁻/nu⁻) mice, the increase in TNF- α and IFN- γ mRNA expression was found to be closely related to a reduction in LECT2 mRNA expression. These results suggest that the reduction in expression of LECT2 mRNA is not directly involved in apoptosis and may be inversely related to the expression of TNF- α and/or IFN- γ mRNA in the injured liver.

Key words leukocyte-derived chemotaxin 2; Con A-induced hepatic injury; apoptosis; LECT2; TNF- α and IFN- γ mRNA

Leukocyte-derived chemotaxin 2 (LECT2) is a novel neutrophil chemotactic factor that has been purified as a 16 kDa protein from the culture medium of activated human T-cell leukemia SKW3 cells after stimulation with phytohemagglutinin. LECT2 mRNA is expressed mainly in hepatocytes of human adult and fetal liver in a constitutive manner.^{1,2)} Differences in the amounts of LECT2 secreted into the culture medium have been observed in various human hepatoma cell lines, including HuH-6, HepG2 and HLF.³⁾ Thus, LECT2 seems to be a functional cytokine involved in the regulation of hepatocyte activity. The human LECT2 gene has been mapped to chromosome 5q31.1-q,⁴⁾ where loci contain a cluster of several genes encoding immuno-regulatory cytokines and transcription factors. These observations have led to the suggestion that LECT2 might be an immunomodulating factor expressed in hepatocytes. In a recent study, a polymorphism in Val58Ile of human LECT2 was demonstrated to be a risk factor for progression of rheumatoid arthritis.⁵⁾

Concanavalin A (Con A) has been reported to cause both direct injury to hepatocytes, mediated by activated CD4⁺ lymphocytes, and hepatocellular apoptosis, induced by tumor necrosis factor (TNF)- α and interferon (IFN)- γ .^{6,7)} The development of Con A-induced hepatic injury can be inhibited by neutralizing anti-TNF- α antibodies.⁷⁾ Con A is often used to prepare experimental animals with high levels of cytotoxic T-lymphocytes, because these cells are involved in the development of viral infections in humans.⁸⁾

The changes in expression of LECT2 mRNA in normal mouse liver,⁹⁾ and the factors that affect LECT2 mRNA expression during liver injury have not yet been elucidated. Therefore, in the present study, LECT2 mRNA expression was investigated during Con A-induced liver injury.

MATERIALS AND METHODS

Materials Con A (Jack bean, type IV, Sigma, St. Louis, U.S.A.) was dissolved in physiological saline (sterile, pyrogen-free, Ohtsuka Pharmaceutical Co., Ltd., Osaka, Japan). Z-Val-Ala-Asp(OMe)-CH₂F (Z-VAD-FMK, Calbiochem, Canada) was initially dissolved in dimethylsulfoxide and then diluted with sterile physiological saline.

Animals Female BALB/c and BALB/c nu⁻/nu⁻ mice (6-week-old) were purchased from Charles River Japan Inc. (Atsugi, Japan) and acclimatized to a room temperature of 23 $\pm$ 3 °C, a humidity of 55 $\pm$ 10%, and a 12 h dark/light cycle for at least 4 d before the experiments. The animals were fed commercial laboratory chow (solid, CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*. All animal experiments were conducted in accordance with the guidelines of the Zeria Pharmaceutical Co., Ltd., Animal Care and Use Committee.

Con A-Induced Hepatic Injury Con A (13 mg/kg) was injected into the mice intravenously (i.v.).⁷⁾ Z-VAD-FMK was injected i.v. on four occasions: 0, 2, 4 and 6 h after Con A injection. Blood was obtained from the orbital vein under ether anesthesia at various intervals (0, 2, 4, 8, 24, 48, 72 and 96 h) after Con A injection and the serum was separated and used for measurement of serum glutamate pyruvate transaminase (s-GPT) activity. The liver was excised and used for the detection of DNA fragmentation and TNF- α , IFN- γ and LECT2 mRNA expression.

s-GPT Activity s-GPT activity was measured colorimetrically (555 nm) using a transaminase CII-test Wako kit (Wako, Osaka, Japan).

Liver DNA Fragmentation Assay DNA fragmentation was measured by quantifying cytosolic oligonucleosome-bound DNA using a cell death detection enzyme immuno-

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bent assay (ELISA), as described previously.¹⁰⁾ Briefly, excised liver was homogenized in a lysis buffer, allowed to stand at room temperature for 30 min, and then centrifuged at $700\times g$ for 10 min. Twenty microliters of supernatant (equivalent to 170 μ g wet weight liver tissue) was used for colorimetric measurement (450 nm) of DNA fragmentation using a cell death detection ELISA^{PLUS} kit (Boehringer Mannheim, Mannheim, Germany).

Western Blotting Analysis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of mouse LECT2 protein in serum was performed as described by Laemmli using 14% gels.¹¹⁾ Proteins were blotted onto a PVDF filter (Millipore Japan, Tokyo, Japan). Purified polyclonal antibodies diluted to 0.2 μ g/ml with phosphate-buffered saline (PBS) containing 3% skimmed milk were laid onto the filter. The filter was incubated for 60 min at room temperature and then washed three times with washing buffer containing 0.1% Tween 20 in PBS. Horseradish peroxidase-protein G was then placed on the filter and allowed to stand for 60 min at room temperature. The filter was washed three times with washing buffer and positive bands were detected with an ECL detection kit (Amersham Pharmacia, Biotech AB, Uppsala, Sweden).

RNA Isolation and Probe Labeling Total RNA was prepared from mouse liver using Isogen (NipponGene, Toyama, Japan), and 1 μ g of RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Takara Shuzo, Kyoto, Japan) in a reaction volume of 20 μ l containing oligodT-adaptor primers.¹²⁾ Polymerase chain reaction (PCR) amplification was performed with GeneAmp 9700 (Perkin-Elmer Foster, CA, U.S.A.), in a reaction volume of 50 μ l containing 1 μ l of DNA solution. The mouse LECT2 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers were as follows: LECT2: TCCTCGAGATGATTCC-CACAACAATC and TGCTCGAGTAGGTATGCTGTGG; G3PDH: TGAAGGTCGGTGAACGGATTGGC and CATGTAGGCCATGAGGTCCACCAC. LECT2 and G3PDH cDNA probes were prepared by second PCR amplification using digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany).

Northern Blotting Analysis RNA samples (20 μ g/lane) were subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde, and then transferred to nylon membranes. The membranes were prehybridized and hybridized with digoxigenin-11-dUTP-labeled cDNA probes.

TNF- α , IFN- γ and LECT2 mRNA Expression in Liver Total liver RNA was amplified by reverse transcriptase-PCR (RT-PCR), electrophoresed on a 2.0% agarose gel and TNF- α , IFN- γ and LECT2 mRNA expression was then examined. In the RT-PCR, TCCTCGAGATGATTCCCAACAATC and TGCTCGAGTAGGTATGCTGTGG were used as the sense and antisense primers, respectively, for LECT2, while ATGAGCACAGAAAGCATGATC and TACAGGCTTGTCACCTCGAATT were used as sense and antisense primers, respectively, for TNF- α . TACTGCCACGGCACAGTCATT-GAA and GCAGCGACTCCTTTTCCGCTTCCT were used as sense and antisense primers, respectively, for IFN- γ . Finally, TGAAGGTCGGTGAACGGATTGGC and CATGTAGGCCATGAGGTCCACCAC were used as sense and antisense primers, respectively, for control G3PDH determination.

Statistical Analysis All results were expressed as the mean $\pm$ standard error (S.E.M.). Significant differences were analyzed using Dunnett's test for multiple group comparisons and Student's *t*-test for comparisons between two groups. The significance of changes in s-GPT activity was determined after conversion of the measured values to natural logarithms. $p < 0.05$ was considered to be statistically significant.

RESULTS

Western Blotting in Serum A 16 kDa protein band which reacted with the polyclonal recombinant mouse LECT2 antibodies was observed in serum from normal mice. The level of LECT2 protein decreased temporarily from 8–24 h after Con A treatment, but recovered after 48 h as s-GPT activity decreased (Figs. 1A, B). The other weaker bands observed were probably due to non-specific background binding or a LECT2-related protein which reacted with the polyclonal antibodies.

S-GPT Activity and DNA Fragmentation during Con A-Induced Liver Injury in Mice A significant increase in s-GPT activity was detected in mice treated with Con A compared with untreated mice. Maximal activity was observed 8 h after treatment, and then decreased to normal levels at 96 h (Fig. 1B). DNA fragmentation in liver tissue was detected from 2 h after Con A injection in a time-dependent manner, as an increase in s-GPT activity was detected in serum. The intensity of DNA fragmentation reached a maximum 8 h after Con A injection, but thereafter fragmentation could not be detected (Fig. 1C). The increase in liver DNA fragmentation was observed earlier than the changes in s-GPT activity (Figs. 1B, C).

Expression of LECT2, TNF- α and IFN- γ mRNA during Con A-Induced Liver Injury in Mice LECT2 mRNA, which was expressed constitutively in normal liver, was de-

Fig. 1. Changes in the Level of LECT2 (A) in Serum and s-GPT Activity (B), and DNA Fragmentation (C) in Liver after Con A-Induced Hepatic Injury in Mice

Con A (13 mg/kg, i.v.) was injected into BALB/c mice. Purified recombinant mouse LECT2 was used for Western blotting analysis of LECT2 with polyclonal antibodies. The results for s-GPT activity and DNA fragmentation are presented as means $\pm$ S.E.M. ($n=6$). ** $p < 0.01$: Dunnett's test (vs. normal).

Fig. 2. Changes in LECT2, TNF- α and IFN- γ mRNA Expression during Con A-Induced Hepatic Injury in Mice

BALB/c mice were killed at various times after Con A (13 mg/kg, i.v.) injection. Liver LECT2 mRNA expression (A) was analyzed by Northern blotting, and TNF- α and IFN- γ mRNA expression (B) was determined by RT-PCR. Immunodeficient *nu*⁻/*nu*⁻ and wild-type mice were killed 8 h after Con A injection, and various cytokines mRNAs (C) were determined by RT-PCR.

tected weakly 8 and 24 h after Con A injection. Expression of LECT2 mRNA was detected clearly again at 48 h, showing recovery of expression (Figs. 2A, B). Expression of TNF- α and IFN- γ mRNAs was not detected in normal mouse liver. However, mRNA of both cytokines was clearly detectable 2, 4 and 8 h after Con A injection. Expression then declined gradually until it became undetectable at 96 h (Fig. 2B). This pattern of expression was the opposite to LECT2 mRNA expression. Neither reduction of LECT2 mRNA nor induction of TNF- α and IFN- γ mRNA expression was observed in immunodeficient *nu*⁻/*nu*⁻ mice 8 h after Con A injection (Fig. 2C).

Effect of Z-VAD-FMK on Con A-Induced Liver Injury in Mice Administration of Z-VAD-FMK i.v., at a dose of 3 mg/kg, inhibited the Con A-induced increase in s-GPT activity in serum, and reduced the intensity of DNA fragmentation in liver 8 h after Con A injection (Figs. 3A, B). The reduction in LECT2 mRNA expression caused by liver injury with Con A was not reversed by administration of Z-VAD-FMK (Figs. 3C, D). Furthermore, induction of TNF- α and IFN- γ mRNA expression at 8 h was not inhibited by Z-VAD-FMK (3 mg/kg, i.v.) (Fig. 3C).

DISCUSSION

In this study, expression of LECT2 mRNA was shown to be reduced temporarily in the early stages of Con A-induced liver injury in mice, and then returned to normal levels as recovery from liver injury occurred. After Con A injection, s-GPT activity and DNA fragmentation in liver increased grad-

Fig. 3. Effect of Z-VAD-FMK on s-GPT Activity, Liver DNA Fragmentation and Expression of LECT2, TNF- α and IFN- γ mRNA after Con A-Induced Hepatic Injury in Mice

s-GPT activity (A) and liver DNA fragmentation (B) were measured 8 h after Con A injection. The results are presented as means $\pm$ S.E.M ($n=6$). ## $p<0.01$: Student's *t*-test. * $p<0.05$: Dunnett's one-tailed test. LECT2, TNF- α and IFN- γ mRNA expression was detected by RT-PCR (C), and/or Northern blotting (D), respectively. Lane 1: normal group; lane 2: control group (Con A injection without Z-VAD-FMK); lane 3: Con A + Z-VAD-FMK.

ually, reached maximum levels at 8 h, and then decreased. Expression of TNF- α and IFN- γ mRNA in liver was detected 2 h, and then reduced 24 h after Con A injection. Reduction of LECT2 mRNA expression could be observed until 4 h after Con A injection, whereas no increase in s-GPT activity or DNA fragmentation was detectable. However, 8–24 h after Con A injection, the expression of LECT2 mRNA was reduced temporarily in the liver, and protein levels of LECT2 in serum paralleled the reduction seen with mRNA. When s-GPT activity had decreased to normal levels, demonstrating recovery from liver injury, expression of LECT2 mRNA was again observed in the liver. In mice with partial hepatectomy, the protein level of LECT2 in the liver was decreased temporarily and re-expressed at 12 h (unpublished observation). Therefore, it was considered that the change in LECT2 mRNA expression in hepatocytes may be related to liver injury, and that re-expression of LECT2 48 h after Con A injection may be due to the increase in production of LECT2 by normal hepatocytes.

TNF- α , IFN- γ and Fas/Fas ligand are known to induce liver apoptosis after Con A-induced liver injury.^{13,14} TNF- α is involved in the triggering of hepatocellular caspase-1, which is followed by apoptosis.¹⁵ The caspase inhibitor, Z-VAD-FMK, prevented Con A-induced liver injury by inhibiting the increase in s-GPT activity and liver DNA fragmentation in the present study. However, TNF- α and IFN- γ mRNA expression observed in the liver after Con A injection was not affected by Z-VAD-FMK. This shows that expression of TNF- α mRNA occurs before the activation of caspase-1. Furthermore, the reduction in LECT2 mRNA expression observed after Con A injection was not prevented by this

reagent. These results suggest that the reduction of LECT2 expression may not be related directly to liver injury caused by the activation of caspase-1, but may be regulated by TNF- α and/or IFN- γ mRNA expression after Con A injection. Tagawa *et al.*¹⁶⁾ demonstrated that IFN- γ knock-out (KO) mice had no hepatitis after exposure to Con A. Moreover, it has been reported that TNF- α is a critical mediator similar to IFN- γ in Con A-induced hepatic injury, and that anti-TNF- α antiserum protected against hepatic injury.^{7,17)} T-cells are known to be involved in Con A-induced liver injury.⁶⁾ Therefore, in our study with immunodeficient mice (BALB/c nu⁻/nu⁻), expression of LECT2 mRNA was not reduced after Con A injection when compared with wild-type mice. These findings suggest that the induction of TNF- α and/or IFN- γ depends on the activation of T-cells and may be related to the reduction in LECT2 mRNA in the injured liver.

In conclusion, the effect of Con A-induced liver injury on neutrophil chemotactic protein, LECT2, is inversely related to the induction of TNF- α and/or IFN- γ mRNA expression, but is not related to caspase-dependent hepatic apoptosis.

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