Dynamic change in expression of LECT2 during liver regeneration after partial hepatectomy in mice

Michiko OHTOMI¹, Hisakazu NAGAI¹, Hideki OHTAKE², Late Toshikazu UCHIDA³ and Kazuo SUZUKI⁴

¹ Department of Biomolecular Science, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510; ² Department of Physiology, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293; ³ Department of Pathology, Nihon University School of Medicine, 30-1 Oyaguchi Kamimachi, Itabashi-ku, Tokyo 173-8610; and ⁴ Department of Bioactive Molecules, National Institute of Infectious Diseases (NIID), 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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ABSTRACT
Leukocyte-cell-derived chemotaxin 2 (LECT2) was first isolated from the culture fluid of phytohemagglutinin-activated human T-cell leukemia SKW-3 cells and was found to be expressed in the human, bovine and murine livers. To further analyze the role of LECT2 in the liver, we investigated the expression of mouse LECT2 (mLECT2) during liver regeneration after partial hepatectomy (PHx) using immunohistochemical and in situ hybridization techniques. Mouse LECT2 protein and mRNA were detected in most hepatocytes in normal mouse; however, at 30 min after PHx, they were not detected in liver tissue. At 2 h after PHx, expression of mLECT2 protein was seen in hepatocytes surrounding the central vein, although mRNA expression levels were still low. At 6 h after PHx, a marked number of hepatocytes expressing mLECT2 protein and mRNA were seen throughout the liver, and at 12 h after PHx, hepatocytes expressing mLECT2 protein and mRNA further increased in number. However, expression levels of mLECT2 protein and mRNA at 24 h after PHx were significantly lower when compared with levels after 12 h. These results indicate that LECT2 triggers the early events of regeneration with concomitant suppression of hepatocyte proliferation.

Liver regeneration after liver injury caused by acute viral infection, parasite infection or liver transplantation is a precisely regulated process during which the surviving cells proliferate to reconstitute the original mass of the damaged organ. Numerous growth factors and cytokines, including epidermal growth factor (EGF), hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and nuclear factor-κB (NF-κB), have been identified in the early-response phase of liver regeneration after partial hepatectomy (PHx) (7, 8, 24), and these have important roles in liver regeneration.

For example, hepatocyte or other damage to the liver induces weakening of intercellular adhesions in experimental mouse and rat models, resulting in a cell cycle shift in hepatocytes from G₀ to G₁ and the production of TNF-α and IL-6 (3, 15, 26). Levels of TGF-α peak at 24 h after PHx, and it acts as an autocrine factor (17, 18). Studies using knockout (KO) mice without NF-κB or signal transducer and activation of transcription 3 (STAT3) showed that NF-κB and STAT3 are required for the initial phase of liver regeneration (2, 4, 5, 11, 26).

The levels of EGF in the liver have been shown to peak at 1 h after PHx, whereas the levels of heparin-binding EGF-like growth factor (HB-EGF) peak at 6 h after PHx (17, 18). Once liver mass has been
regained, termination of hepatocyte cell proliferation is associated with the production of activin, a molecule with autocrine activity (7). Thus, TNF-α, IL-6, TGF-α, NF-kB and HB-EGF all appear to be important molecules for liver regeneration. However, it has been demonstrated that IL-6 is not always essential for the regeneration of liver tissue. For example, STAT3 levels are significantly reduced after hepatectomy in IL-6 mice, and liver mass in IL-6 KO mice completely recovered in the long term (3).

Leukocyte-cell-derived chemotaxin 2 (LECT2) was first isolated by Yamagoe et al. (29) as a novel chemotactic factor for neutrophils derived from the culture fluid of phytohemagglutinin-activated human T-cell leukemia SKW-3 cells. LECT2 is a 16-kDa protein consisting of 133 amino acid residues, and is expressed preferentially in human adult and fetal liver cells (28), human tissues including vascular epithelial cells and smooth muscle cells of the soft tissue surrounding lymph nodes, nerve cells, the bronchial wall and skeletal muscle cells (19). On biopsy analysis, it has been shown that the LECT2 protein is strongly expressed around the central vein with a gradient from the portal tract in human liver tissue (14, 27). LECT2 is identical to chondromodulin II, which stimulates proliferation of chondrocytes and osteoblasts (13, 23), and is homologous to mim-1 (myb-induced myeloid protein 1), a chicken protein whose function remains unknown (6, 20). Although LECT2 was originally demonstrated to have a chemotactic function in vitro, later reports showed that LECT2 is involved in a further multifunctional role in the liver, extending from cell growth, differentiation, damage/repair processes and carcinogenesis to autoimmune diseases (21, 22, 25, 27). However, the function of LECT2 in vivo remains unclear.

In the present study, to further analyze the role of LECT2 in the liver, we investigated the expression of mLECT2 protein and mRNA during liver regeneration after PHx over a 24-hour period using immunohistochemical and in situ hybridization techniques.

MATERIALS AND METHODS

Partial hepatectomy. Male ddY mice (11-week-old) were purchased from Saitama Experimental Animal Supply Co., Ltd. (Saitama, Japan). Each mouse was subjected to 70% PHx according to the method of Higgins and Anderson (12). Mice were sacrificed at various time points after PHx and liver tissue samples were harvested from the remaining posterior lobes. As a control, sham surgery was performed by subjecting mice to midventral laparotomy and closure, followed by removal of the liver at various time points after surgery (laparotomy).

All animal experiments were performed in accordance with the ethical standards of the Animal Research Committee for Animal Experimentation of Toho University.

Immunohistochemistry. Liver tissue samples were fixed with 10% formaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4), and embedded in paraffin. Liver tissue was sliced into 4-µm sections and mounted on slides. Deparaffinized sections were incubated with anti-human LECT2 polyclonal antibody (kindly provided by Dr. Satoshi Yamagoe, National Institute of Infectious Diseases, Tokyo) at 4°C overnight, and subsequently with goat anti-rabbit immunoglobulin G (IgG) conjugated with peroxidase for 1 h at 37°C. Reaction products in each liver tissue section were visualized by treatment with 3,3'-diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin. Sections were rinsed with PBS after each step. As a blocking control, the first antibody was allowed to react with its antigen for 1 h at 37°C, and the mixtures were separated by centrifugation and applied to the sections. Other controls replaced the first antibody with normal rabbit serum and eliminated the first antibody reaction.

In situ hybridization. Liver tissue was fixed with 4% paraformaldehyde in 0.1 M PBS at 4°C overnight, and was then immersed in a series of sucrose solutions (twice in 15% sucrose-PBS for 2 h and once in 20% sucrose-PBS overnight) at 4°C. Tissues were covered with Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN, USA) and were subsequently frozen. Sections (10 µm) were cut on a cryostat microtome at −30°C and were mounted on bovine serum albumin (BSA)-coated slides. cRNA probes were generated from a cDNA fragment encoding mLECT2 (834 bp). The mLECT2 DNA fragment was subcloned into pBluescript II KS(−) at the BamHI sites. Both sense and antisense probes were transcribed from linearized plasmids using a DIG-RNA labeling kit (Roche Molecular Biochemicals Indianapolis, IN, USA). The prehybridization mixture consisted of an aqueous solution of 0.3 M NaCl, 20 mM Tris·HCl (pH 8.0), 50% deionized formamide, 10% dextran sulfate, 1 × Denhardt’s solution, 5 mM EDTA (pH 8.0) and 0.5 mg/mL E. coli tRNA in DEPC-treated water. Sections were hybrid-
ized in hybridization solution containing 0.5 μg/mL sense or antisense probe in a moisture chamber at 50°C for 17 h. Following incubation, sections were washed twice in 50% formamide/2× SSC at 50°C for 20 min followed by 0.5 M NaCl in 10 mM Tris-HCl (pH 8.0) at 37°C for 10 min. Sections were then treated with 20 μg/mL RNase A (0.5 M NaCl in 10 mM Tris-HCl (pH 8.0)) for 10 min at 37°C. RNase-treated slides were subjected to a series of washes (0.5 M NaCl in 10 mM Tris-HCl (pH 8.0) for 10 min at 37°C, 2× SSC containing 50% formamide for 20 min at 50°C, and 1× SSC containing 50% formamide for 20 min at 50°C). DIG-labeled RNA was detected using a DIG-nucleic acid detection kit (Roche Molecular Biochemicals Indianapolis) according to the manufacturer’s instructions.

RESULTS

Expression of mLECT2 protein in the liver after PHx

Using anti-human LECT2 (hLECT2) polyclonal antibody, we monitored mLECT2 protein expression in the livers of mice for 24 h after PHx. In normal liver tissue, most of hepatocytes were diffusely stained with anti-hLECT2 polyclonal antibody (Fig. 1A). Expression level of mLECT2 protein was slightly high in hepatocytes around the central veins compared with those around the periportal vein. At 30 min after PHx, most hepatocytes were unstained, and the only staining was seen in hepatocytes surrounding the central veins, but not the portal veins (Fig. 1B). At 2 h after PHx, expression of mLECT2 protein increased in the hepatocytes surrounding the central veins, showing “perivascular islands” (Fig. 1C). At 6 h after PHx, the number of hepatocytes expressing mLECT2 protein was further increased, and numerous hepatocytes expressed mLECT2 protein, with the exceptions being the single-layered cells surrounding the central veins (Fig. 1D). At 12 h after PHx, the expression of mLECT2 protein increased and was clearly evident in most hepatocytes, but again, the exceptions were the single-layered cells surrounding the central veins (Fig. 1E). However, at 24 h after PHx, expression of mLECT2 protein in hepatocytes was markedly reduced (Fig. 1F).

Expression of mLECT2 mRNA in the liver after PHx

The expression profile of mLECT2 mRNA at each time point after laparotomy was similar to that observed in the normal mouse liver; mLECT2 mRNA was expressed throughout the liver tissue. As shown in Fig. 2A, most of the hepatocytes in normal mice expressed mLECT2 mRNA throughout the liver, while no mLECT2 mRNA expression was observed in negative controls (Fig. 2B). After PHx, the expression profile of LECT2 mRNA was largely similar to that of mLECT2 protein. At 20 min after PHx, mLECT2 mRNA expression levels were markedly reduced, and at 30 min after PHx, little mLECT2 mRNA expression could be detected (Fig. 2C, D), which differed from the livers of normal mice. At 2 h after PHx, mLECT2 mRNA expression was slightly detected in the hepatocytes surrounding the central veins (Fig. 2E). At 6 h after PHx, mLECT2 mRNA expression spread to the hepatocytes around the central veins (Fig. 2F). At 12 h after PHx, the number of cells expressing mLECT2 mRNA expression had increased, and they were distributed throughout the liver (Fig. 2G). However, at 24 h after PHx, the expression of mLECT2 mRNA in hepatocytes was reduced (Fig. 2H), and showed a similar expression profile as mLECT2 protein at 24 h after PHx.

DISCUSSION

In the present study, we examined the expression of mLECT2 in mouse liver over a 24-hour period after PHx by immunohistochemical and in situ hybridization techniques. In the normal mouse liver (0 min, immediately prior to heptectomy), most hepatocytes expressed mLECT2 protein and mRNA. The expression profile for mLECT2 protein in normal mouse liver was similar to that reported for hLECT2 in the human liver (19, 25, 27). During liver regeneration, the observed changes in mLECT2 expression were similar for protein and mRNA levels; expression disappeared in the early stages after PHx and almost fully recovered at 12 h after PHx, but decreased again at 24 h after PHx. Expression level of mLECT2 in normal liver was high in hepatocytes surrounding the central veins and low in hepatocytes surrounding the portal veins. This indicates that LECT2 is an effective coordinator of liver regeneration in the mouse liver. This is because proliferation of hepatocytes in the regenerating liver is initiated around the portal vein and spreads with blood flow, reaching maximum levels around the central vein at 36 to 48 h after PHx.

It has been reported that DNA synthesis levels in mouse hepatocytes in regenerating liver peak at about 24 h after PHx. Subsequently, hepatocytes in the residual tissue divide once or twice during regeneration and the liver tissue recovers its original
and protein increased at 12 h after PHx, reaching levels similar to those prior to PHx. However, at 24 h after PHx, expression levels were reduced. Expression of mLECT2 thus changed markedly and was localized within the liver in the early stages of liver regeneration following PHx. Furthermore, the expression of mLECT2 protein at 30 min after PHx was specifically observed in only a single layer of hepatocytes surrounding the central vein. The numbers of hepatocytes expressing mLECT2 protein at 2 h after PHx increased slightly around the central vein. However, at 6 h after PHx, expression of function at 5 to 7 days after PHx. Original mass and volume was regained at 10 days after PHx (10, 17). In addition, proliferation of hepatocytes is known to be initiated in the area surrounding the portal vein in the direction of blood flow, which then peaks surrounding the central vein at 36–48 h after PHx (1, 16). The present results demonstrate that very little, if any, mLECT2 mRNA and protein were present in hepatocytes at 30 min after PHx, but that from 2 to 6 h after PHx, the number of hepatocytes expressing mLECT2 mRNA and protein increased gradually throughout the liver. Expression mLECT2 mRNA and protein increased at 12 h after PHx, reaching levels similar to those prior to PHx. However, at 24 h after PHx, expression levels were reduced. Expression of mLECT2 thus changed markedly and was localized within the liver in the early stages of liver regeneration following PHx. Furthermore, the expression of mLECT2 protein at 30 min after PHx was specifically observed in only a single layer of hepatocytes surrounding the central vein. The numbers of hepatocytes expressing mLECT2 protein at 2 h after PHx increased slightly around the central vein. However, at 6 h after PHx, expression of

**Fig. 1** Changes in mLECT2 expression in regenerating livers after PHx, as determined by immunohistochemical staining for mLECT2. mLECT2-positive signals in hepatic parenchymal cells are light brown. The pale blue dots denote hepatocyte nuclei. Cv denotes the central vein, P the periportal vein. A, 0 min; B, 30 min after PHx; C, 2 h after PHx; D, 6 h after PHx; E, 12 h after PHx; F, 24 h after PHx. Scale bars = 200 µm.
mLECT2 protein was observed in most hepatocytes, except for those in a single layer surrounding the central vein. These results indicate that LECT2 might play an important role in the early events, such as cell proliferation, of liver regeneration after PHx.
It has also been reported that some cytokines regulate proliferation during the initial period of liver regeneration. TNF-α, which triggers liver regeneration, is secreted from Kupffer cells in the liver and subsequently activates NF-κB production in hepatocytes. IL-6 is also secreted from Kupffer cells and activates the production of another transcription factor, STAT3, in hepatocytes. Thus, STAT3 is activated in the early stages by TNF-α and IL-6 (8, 9, 17, 18). Activation of STAT3 is observed at 5 h after PHx, which indicates a shift in the cell-cycle phase from G₀ to G₁ at about 4 h after PHx (7, 8, 9). In the present study, expression of mLECT2 decreased at 30 min after PHx, returned to original levels at 2–12 h after PHx, before decreasing again at 24 h after PHx. This suggests that, in contrast to the roles of TNF-α and IL-6, the role of mLECT2 may be to regulate the proliferation of hepatocytes. In addition, the role of mLECT2 in liver regeneration may be related to those of initiation factors such as TNF-α and IL-6, as mLECT2 is secreted from hepatocytes, while TNF-α and IL-6 are secreted from Kupffer cells.

The role of LECT2 in liver regeneration may also be related to those of growth factors, as LECT2 may regulate the proliferation of hepatocytes by a suppressive action. HGF, EGF and HB-EGF are well-known growth factors for hepatocytes. Levels of HGF in plasma increased by a factor of 20 at 1 h after PHx, returned to original levels within 24 h, and then increased again at 72 h after PHx. HGF mRNA peaked in non-hepatocytes at 24 h after PHx. In addition, after hepatectomy, levels of EGF in plasma increased slightly and stimulated the proliferation of hepatocytes at 1 h after PHx. Levels of HB-EGF peaked in non-hepatocytes at 6 h after PHx, and was associated with the proliferation of hepatocytes (8). In the present study, a marked change in the distribution of mLECT2 was observed during the initial period of liver regeneration after PHx, which indicates that, together with growth factors, mLECT2 contributes to liver regeneration by triggering the early events of regeneration while suppressing hepatocyte proliferation. In the present study, the absence of cells expressing mLECT2, except for hepatocytes surrounding the central vein 30 min after PHx, supports the notion that proliferation of hepatocyte starts in regions surrounding the portal vein and then expands towards the central vein.

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