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Expression of a Neutrophil Chemotactic Protein LECT2 in Human Hepatocytes Revealed by Immunochemical Studies Using Polyclonal and Monoclonal Antibodies to a Recombinant LECT2

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A recombinant human neutrophil chemotactic protein LECT2 (rhLECT2) was purified as a 16-kDa protein from the culture fluids of stable transfectants derived from CHO cells (clone C1D8-1) and L929 cells (clone L2E4-1). The N-terminal amino acid sequence of the protein secreted by both clones were homologous to the previously described bovine LECT2. We produced polyclonal and monoclonal antibodies against rhLECT2 and investigated secretion of LECT2 protein in six human hepatoma cell lines, which express LECT2 mRNA, and in hepatocytes of normal human livers by a sandwich enzyme-linked immunosorbent assay and by immunostaining using the antibodies, respectively. We revealed that five of six hepatoma cell lines secreted LECT2 into culture fluids at concentrations of 30-135 ng/mg. We also demonstrated that the cytoplasm of human hepatocytes was diffusely stained, although periportal hepatocytes tended to be weakly and granularly stained by immunostaining. These results indicated that the novel protein was expressed in hepatocytes and suggested an important role of LECT2 in the cells in addition to the activation of neutrophils.

Neutrophil chemotactic factors stimulate neutrophils in response to increasing concentrations of the factors released from inflammatory sites. Neutrophils stimulated by factors are recruited from the blood through the vessel wall to the inflammatory site (1). The chemotactic factors, which are released from inflammatory sites play an important role in activating neutrophil functions, IL-8 and other chemotactic factors have been isolated (2-5). Recently a novel neutrophil chemotactic factor LECT2 has been purified from the culture fluid of the activated T-cell leukemia SKW-3 cells by PHA stimulation (6, 7).

Some of the chemotactic factors have been demonstrated to exert functions *in vivo* other than the original activity demonstrated *in vitro.* Chemokines have diverse cellular effects such as changing in the proliferation rate and expression of cell surface proteins as well as inflammatory mediators (8). For example, CC-chemokines show anti-viral activity against human immunodeficiency virus through their cellular receptors on T cells (9-11). Thus, some chemotactic factors and the related inflammatory cytokines are not always involved only in the inflammatory process. Although LECT2 has been originally demonstrated to have a chemotactic function *in vitro*, biological function *in vivo* has not been thoroughly clarified.

In the present study, we performed to identify the cells expressing LECT2 protein leading to its physiological role as we obtained data that LECT2 transcript was abundantly and almost exclusively detected in the liver and hepatoma cell lines (submitted). We analyzed LECT2 expression in human livers by immunochemical techniques using the antibody against recombinant LECT2 (rhLECT2) from mammalian cells transfected with hLECT2 expression vector.

MATERIALS AND METHODS

Establishment of mammalian cells expressing rhLECT2. 5′-end of hLECT2 cDNA in the pOLECT2 plasmid was deleted until up 5-

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Abbreviations: LECT2, leukocyte cell-derived chemotaxin 2; PO, horseradish peroxidase; TFA, trifluoroacetic acid.

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bp from the predicted ATG by exonuclease III, treated with Klenow fragment, ligated to a PstI linkers, and digested with BamHI and PstI restriction enzymes. The obtained hLECT2 cDNA fragment was cloned into the PstI and BamHI site of an expression vector pcD-SR α 296, which was gifted by Dr. Takebe in this Institute. The obtained hLECT2 expression vector was named pcD-SR α 296. CHO cells and L929 cells grown to ~50% confluence were transfected with 16 μg of the pSR α LECT2 and 1 μg of the pRSVneo per 10-cm dish by Lipofectamine (BRL). The G418 resistant cells were selected at 800 μg/ml for CHO and 400 μg/ml for L929 to obtain stable transfectants.

Screening of transfectants expressing rhLECT2. Stable transfectants expressing rhLECT2 were screened by SDS-PAGE for 16-kDa protein. Various stable transfectants derived from CHO cells and L929 cells were incubated with 50 μl MEM medium free of both methionine and fetal bovine serum (FBS), and contained 200 μCi/ml ^{35}S-methionine (NEN) in the early stationary phase in a 96-well plate for 8 hrs in a CO_2 incubator at 37°C. The culture fluid was separated by a SDS-PAGE with 15% gel and analyzed with an Image Analyzer BAS2000 (Fuji Film Co. Ltd., Tokyo).

Purification of rhLECT2. CHO cells and L929 cells were maintained in MEM medium supplemented with 10% FBS. rhLECT2 was purified from the culture fluids of two stable transfectant cell clones C1D8-1 and L2E4-1 derived from CHO cells and L929 cells, respectively. The cells were cultured until confluent at 37°C in a 5% CO_2 incubator in the culture plates Serutorei (500 cm^2, Sumitomo Bakelite, Tokyo, Japan), harvested into a 500-ml plastic bag, and stored at −80°C until use. rhLECT2 was purified by the procedures which were essentially the same as those for native LECT2 described previously (6). Hydroxylapatite HPLC was carried out by a linear gradient of 0.1M-0.4M sodium phosphate buffer (pH 7.4) at a flow rate of 0.75 ml/min.

Preparation of monoclonal antibodies and polyclonal antibody to rhLECT2. Twenty-five μl of a mixture containing rhLECT2 (2 mg/ml) and complete Freund's adjuvand (1:1) was injected into the foot pad of BALB/c mice, then, rhLECT2 emulsified in incomplete Freund's adjuvand was injected into the mouse (15 μg/mouse) at 4 and 7 days. Three days later of the final injection lymphonode cells were fused with cells of myeloma PAI at a ratio of 5 to 1 by polyethyleneglycol-400 procedure or at a ratio of 2 to 1 by the electric fusion procedure (12, 13). After cloning the hybridomas, the monoclonal antibodies were purified by a Protein G Sepharose column chromatography of the peritoneal fluids of mice. Polyclonal antibody to rhLECT2 was also prepared. A rabbit was subcutaneously injected with 50 μg of rhLECT2, and then 200 μl of mixture containing rhLECT2 (0.5 mg/ml) and complete Freund's adjuvand (1:1) was injected into the back of the rabbit every week. The polyclonal antibody was purified from the serum by Protein A Sepharose column chromatography at five weeks after the final injection.

Western blotting analysis. SDS-PAGE of rhLECT2 was performed by Laemmli's method (14) with 15% gel. The proteins were blotted to PVDF filter (Millipore Japan, Tokyo). The purified monoclonal antibodies diluted to 2 μg/ml with PBS containing 5% skim milk was laid on the filter. The filter was incubated for 60 min at room temperature, and then washed three times with a washing buffer containing 0.05% Tween 20 in PBS. After that biotinylated anti-mouse Ig was laid on the filter and it was kept 60 min. This filter was then incubated with peroxidase-streptoavidin conjugate (Amersham, U.S.A.) The filter was washed three times with the washing buffer, and then the positive band was determined with a SuperSignal™ ULTRA Chemiluminescent Substrate (Pierce, Rochford, U.S.A)

Measurement of LECT2 in culture fluids of human hepatoma cell lines. The cell lines HuH-6, HuH-7, HLE, HLF, PLC/PRF/5 and HepG2, which were provided by the Japanese Cancer Research Resources Bank, were maintained in MEM medium supplemented with 10% FBS. The culture fluids of the hepatoma cell lines were assayed for the secretion level of LECT2. Samples (100 μl) were plated to the well coated with monoclonal antibody (G2A5), and kept for 1 hr at room temperature, and the plate was washed four times with PBS containing 0.5% Tween 20. Then, 100 μl of horseradish peroxidase-labeled (PO)-Fab' solution (3 μg/ml) of polyclonal anti-rhLECT2 antibody was prepared (15) and added to the well, and kept for 1 hr at room temperature, and the plate was washed four times with PBS containing 0.5% Tween 20. Then, 100 μl of the substrate solution containing tetramethylbenzidine and hydrogen peroxide was added to the well and the reaction was terminated by addition of 100 μl of 1N phosphoric acid. The plate was measured with a plate reader at 450-650 nm. The LECT2 amounts were calibrated with a standard curve of rhLECT2. Protein amount of each hepatoma cell line was determined using the supernatant of cell lysate prepared with the lysis buffer, pH 7.5, containing 50 mM HEPES, 0.1% Tween-20, 150 mM NaCl, 1 mM EDTA and 2.5 mM EGTA.

Immunostaining of LECT2 in the liver. Two formalin-fixed and paraffin-embedded liver needle biopsies were processed for following indirect immunoperoxidase methods as described previously (16). These liver biopsies were obtained from a 32-year-old male and 43-year-old female for histological examination because they were positive for anti-hepatitis C virus antibody. In spite of positive serum antibody, the patients were judged not to be infected with hepatitis C virus by negative immunostaining for this virus (11). Histologically, these livers were demonstrated to be nearly normal. Briefly, deparaffinized sections, 4 μm in thickness were incubated with an anti-LECT2 polyclonal antibody or each of two monoclonal antibodies (G2A5 and 89F2) at a dilution of 1:500 at 4°C overnight, and with goat anti-rabbit IgG in the case of polyclonal antibody or goat anti-mouse IgG in the case of monoclonal antibody, conjugated with PO (Medical & Biological Labs. Co. Ltd., Nagoya, Japan) at a dilution of 1:500 for 1 h at 37°C. The reaction products were visualized by treatment with 3,3′-diaminobenzidine tetrahydrochloride, and the sections were further briefly counterstained with hematoxylin. After each step, the sections were rinsed with PBS. As a blocking control, the fist antibodies were absorbed with its antigen (45 μg of antigen in 1 ml of each antibody diluted by 1:500) for 1h at 37°C, followed by clarification by centrifugation, and applied to the sections. Other controls include replacement of the first antibody with normal rabbit serum and elimination of the first antibody reaction.

RESULTS

Expression of rhLECT2 in mammalian cells. Various stable transfectants derived from CHO cells and L929 cells transfected with LECT2 cDNA expression vector were screened out. Six stable transfectants C1D8-1, C2A6 and C2B5-1 derived from CHO and L1B2-1, L2E4-1 and L2F8-5 from L929 highly expressed a 16-kDa protein (Fig. 1A). These six cell clones mostly produced the 16-kDa protein into the culture supernatants, but non-transfected CHO cells and L929 cells were not detected for the 16-kDa protein. The clones C1D8-1 and L2E4-1 cells produced the protein at the highest levels.

Purification of rhLECT2 from transfectants. We purified the 16-kDa protein from the culture fluids of the C1D8-1 and L2E4-1 cell. Purification procedures followed the method used for purification of native LECT2; DEAE-Sepharose and CM-Sepharose column chromatographies (6). The 16-kDa proteins were completely purified by the CM-Sepharose column rechromatography (Fig. 1B). The elution profile of sample

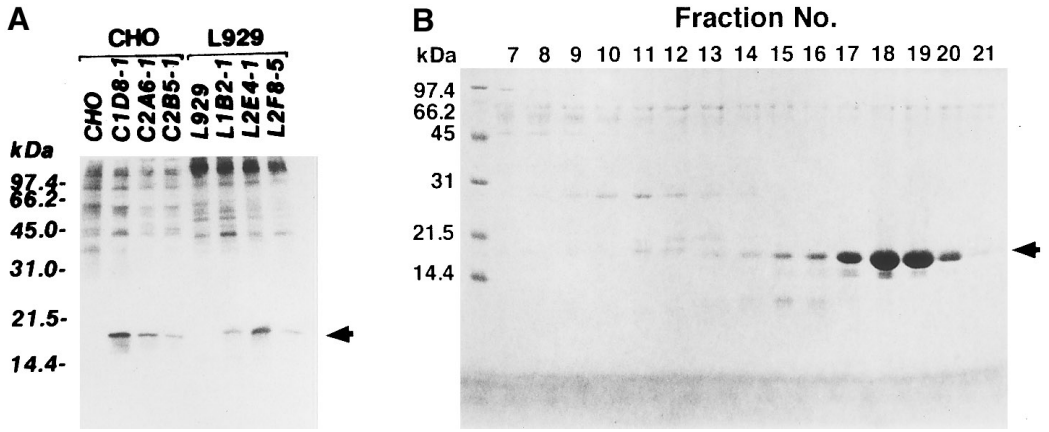

FIG. 1. rhLECT2 expression in stable transfectants of CHO and L929 cells. A, Screening of transfectant clones expressing rhLECT2. The culture fluid (10 μl) was analyzed by a 15% SDS-PAGE using an Image Analyzer BAS 2000. B, SDS-PAGE of rechromatography of CM-Sepharose column in the purification of rhLECT2 from culture fluid of CHO C1D8-1 cells.

derived from C1D8-1 cells was almost identical to that from L2E4-1 cells. The protein from the CM-Sepharose chromatography step yielded 22 mg protein from 8 liters culture fluid of C1D8-1 cells and 1.6 mg from 4 liters of L2E4-1 cells, respectively. The N-terminal amino acid sequence of 16-kDa protein was determined as GPWANIxAG, indicating the homologous sequence to previously determined sequence of bovine LECT2 (bLECT2) after cleaved at the site between Ala-18 and Gly-19 to eliminate the signal sequence (6). The amino acid sequences of N-terminus of the proteins from C1D8-1 cells gave two peaks by HPLC on a hydroxylapatite column chromatography were identical (data not shown). Moreover, the molecular mass of rhLECT2 in the peaks was identical on SDS-PAGE.

Specificity of monoclonal antibody and polyclonal antibody to rhLECT2. We determined the reactivity of monoclonal antibodies A1G1, H12D10, G2A5, and 89F2 with rhLECT2 by Western blotting analysis. As shown in Fig. 2, three monoclonal antibodies specifically reacted with rhLECT2. The monoclonal antibodies H12D10, G2A5 and 89F2 similarly reacted with rhLECT2 with the same level, whereas A1G1 weaker. In addition, we determined the subclass of the monoclonal antibodies of rhLECT2 as follows; A1G1C6 and G2A5 were IgG2b, and H12D10 was IgM.

Secretion of LECT2 from hepatoma cell lines. As the mRNA is mainly expressed in human liver and detected in human hepatoma cell lines (submitted), the antibodies were applied to quantify LECT2 concentration in the culture fluid of various human hepatoma cell lines. We measured LECT2 secreted into the culture fluid of human hepatoma cell lines HuH-6, HuH-7, HLE, HLF, PLC/PRF/5 and HepG2 by ELISA method (Fig. 3). HuH-6 cells showed the highest secretion (135 ng/mg protein), and HuH-7 cells and HLF cells showed the lowest secretion (30 ng/mg protein). LECT2 was not detected in HLE under these conditions. The differences in concentration of hLECT2 in the culture fluids of these six hepatoma cell lines were parallel to mRNA expressions (submitted).

Immunostaining of the liver. Based on the secretion of LECT2 from human hepatoma cell lines parallel with the mRNA expression, we applied the antibodies to the histological examination of hLECT2 in human liver biopsy. As shown in Figs. 4A-C, the polyclonal antibody gave positive staining of the two human livers. All the hepatocytes showed nearly diffuse positive staining within the cytoplasm in a homogenous pattern, although some periportal hepatocytes tended to

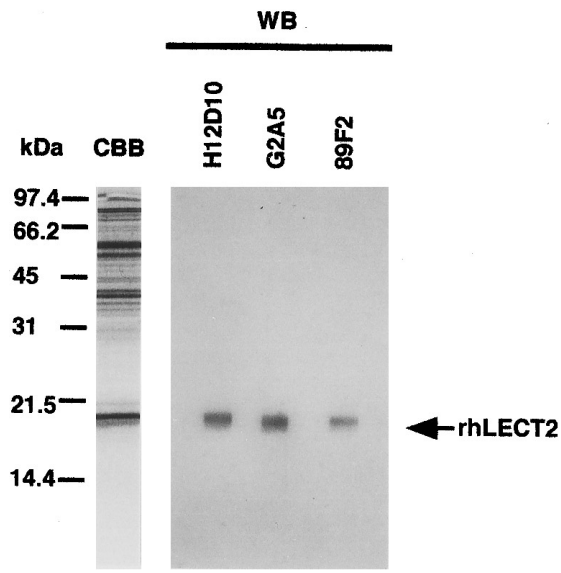

FIG. 2. Western blotting of rhLECT2 with monoclonal antibodies. Reactivity of four monoclonal antibodies H12D10, G2A5, and 89F2 were tested by Western blot using purified rhLECT2 (1 μg/lane) and E. coli lysate (JM109). CBB, Coomassie brilliant blue staining.

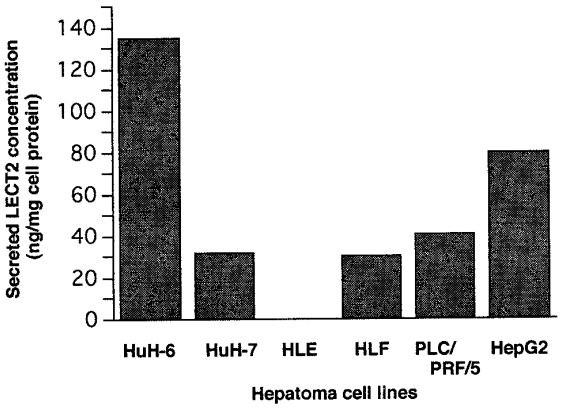

FIG. 3. Expression and secretion of LECT2 from hepatoma cell lines. Concentrations of LECT2 in the culture fluids were measured by the sandwich ELISA system. Cellular protein represents the concentration of total cell lysate as described in Materials and Methods.

exhibit somewhat weaker and granular staining. Although the staining patterns obtained with polyclonal and one monoclonal (89F2) were the same, the staining intensity was much weaker for monoclonal antibody than for polyclonal antibody (data not shown). Except for the hepatocytes, the endothelial cells of hepatic arteries and portal veins of portal tracts and central veins were positively stained for LECT2 (Fig. 4C). The blocking control and other controls abolished positive staining of hepatocytes (data not shown), thus confirming that the staining is specific to LECT2. The monoclonal antibody G2A5 gave the negative staining result.

DISCUSSION

In the present study, we obtained six stable transfectants of CHO and L929 cells producing and excreting rhLECT2 mainly into the culture fluids. The amino acid sequence of N-terminus of rhLECT2 purified from the culture fluids from the two clones C1D8-1 and L2E4-1 contained the sequence of a cleavage site between Ala-18 and Gly-19 to eliminate the signal sequence and was homologous to that of the purified bLECT2 described previously (6). This supports that the molecular mass of rhLECT2 is almost identical to native bLECT2 on SDS-PAGE (6). This strongly suggests that native mature hLECT2 is secreted from cells after cleaved at the same site.

In order to examine the physiological role of hLECT2 *in vivo* we prepared both monoclonal and polyclonal antibodies to rhLECT2 in the present study. Consistent with LECT2 mRNA expression in human liver (submitted), we demonstrated that most hepatoma cell lines expressed LECT2. The concentrations of LECT2 secreted in the culture fluids of the six hepatoma cell lines showed parallel to the mRNA expressions. Further, the formalin-fixed and paraffin-embedded specimen from liver biopsy was specifically reacted with the antibodies. These results strongly suggest that LECT2 produced in hepatocytes is secreted *in vivo*. Furthermore, the LECT2 revealed to localize in some endothelial cells of hepatic arteries, portal vein and central veins by immunostaining, but these contents were much less

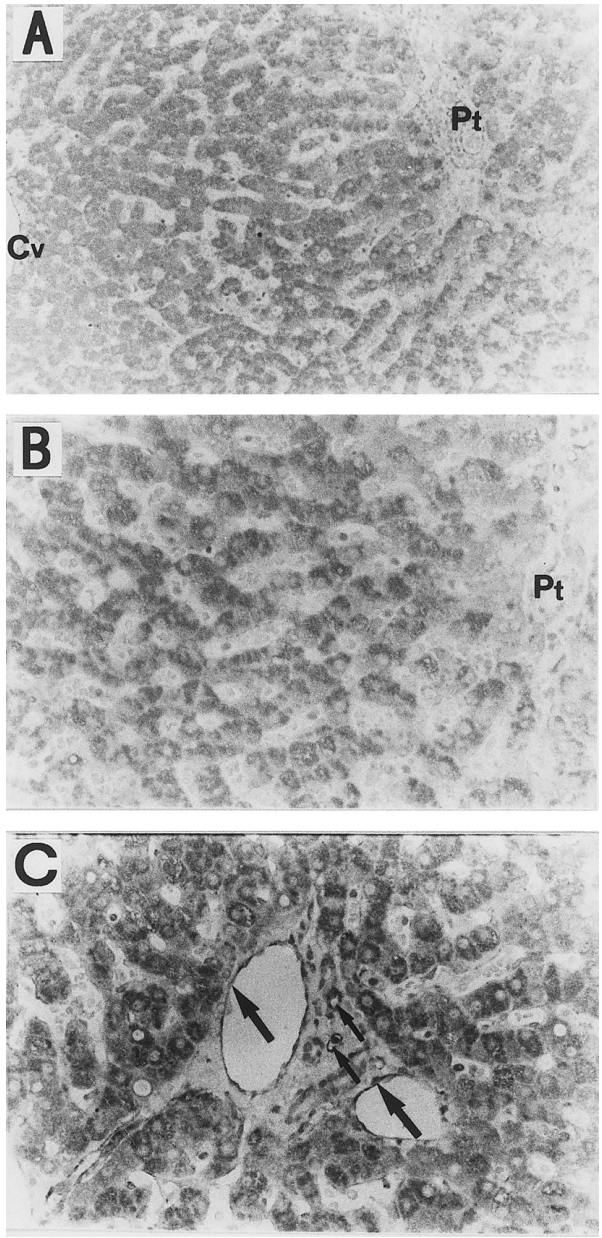

FIG. 4. Distribution of LECT2 in liver tissue. Histological observations of LECT2 in human liver by immunostaining technique using polyclonal antibody to rhLECT2. The formalin-fixed and paraffin-embedded specimen from liver biopsy was stained. Pt, portal trait; Cv, central vein. A, ×100; B, ×200; C, the hepatic arteries (small arrows) and the portal veins (large arrow) exhibit positive linear staining along the endothelial lining. The hepatocyte surrounding the portal tracts show positive staining within he cytoplasm, weakly counterstaining by hematoxylin, ×200.

than those of hepatic cells. The endothelial cells of sinusoids are free of LECT2 immunostaining. The significance of endothelial localization of above three vessels in addition to hepatocyte awaits further investigation.

Concerning the role of LECT2 *in vivo*, this protein may take part in another important physiological role in the human livers in addition to chemotactic activity *in vitro*. In normal liver, LECT2 is expressed, but neutrophil infiltration has not been observed, suggesting that LECT2 may not be chemotactic protein *in vivo* by unknown reasons. The other hand, in liver disease such as Mallory bodies in alcoholic liver disease (17) and ductural transformation of hepatic cords associated with periportal hepatocellular loss (18) without bacterial infection, neutrophilic infiltration in liver has been observed. In these cases, LECT2 or other chemotactic factor such as IL-8 may act in the tissue. The relation of LECT2 or other chemotactic factor(s) with these diseases remains to be clarified.

Our present finding that LECT2 is expressed and secreted from liver hepatocytes *in vivo* may suggest a novel physiological function of this protein in addition to the original activity to neutrophils *in vitro*.

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