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Hematopoiesis-dependent expression of CD44 in murine hepatic progenitor cells

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ABSTRACT

The fetal liver serves as the predominant hematopoietic organ until birth. However, the mechanisms underlying this link between hematopoiesis and hepatogenesis are unclear. Previously, we reported the isolation of a monoclonal antibody (anti-Liv8) that specifically recognizes an antigen (Liv8) present in murine fetal livers at embryonic day 11.5 (E11.5). Liv8 is a cell surface molecule expressed by hematopoietic cells in both fetal liver and adult mouse bone marrow. Here, we report that Liv8 is also transiently expressed by hepatoblasts at E11.5. Using protein purification and mass spectrometry, we have identified Liv8 as the CD44 protein. Interestingly, the expression of Liv8/CD44 in fetal liver was completely lost in *AML1*$^{-/-}$ murine embryos, which lack definitive hematopoiesis. These results show that hepatoblasts change from Liv8/CD44-negative to Liv8/CD44-positive status in a hematopoiesis-dependent manner by E11.5, and indicate that Liv8/CD44 expression is an important link between hematopoiesis and hepatogenesis during fetal liver development.

Fetal liver is known to serve as the predominant hematopoietic organ during its own proliferation and differentiation until birth [1]. Hepatogenesis and hematopoiesis have close interactions as follow. Hepatoblasts, which are hepatic progenitor cells, are thought to support hematopoiesis because hepatocyte-like cell lines were reported to be able to support hematopoiesis [2,3]. On the other hand CD45-positive (CD45^{+}) hematopoietic cells in the fetal liver produce an Interleukin-6 (IL-6) family cytokine, Oncostatin M (OSM), to promote the development of hepatocytes in the middle to late liver development [4,5]. However, the molecules linking hepatogenesis and hematopoiesis during early fetal liver development remains unknown.

The process of embryonic liver development can be divided into several distinct stages [6]. The liver primordium proliferates and invades the septum transversum mesenchyme to give rise to the hepatic codes and buds at E9.5. Hepatic cells at this stage, called hepatoblasts, possess the potential to differentiate into both parenchymal hepatocytes and bile duct epithelial cells. At around E10.5, hematopoietic stem cells originating from aorta–gonad–mesonephros (AGM) region colonize the fetal liver and expand their mass and lineage diversity, such as erythrocytes [1,7]. Hepatoblasts participate in creating the hematopoietic microenvironment in concert with other stromal cells to promote embryonic hematopoiesis [2,3].

We have prepared several monoclonal antibodies specifically recognizing murine fetal livers because molecular markers and tools were needed to understand the mechanisms of early liver development [8,9]. One of the antibodies, called anti-Liv2,

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recognized hepatoblasts specifically at E9.5–E12.5. We could analyzed hepatoblast proliferation in knockout mice using anti-Liv2 [9,10]. Furthermore, we have reported another antibody, anti-Liv8, that recognize both hematopoietic progenitor cells in fetal liver at E11.5 and CD45$^+$ hematopoietic cells in adult bone marrow [11,12]. However, its molecular identification of the Liv8-antigen (Liv8) and its expression in embryonic development were remained unknown.

In this report, we show a molecular identification of Liv8 as CD44. Our finding suggest that Liv8/CD44 links hematopoiesis and hepatogenesis by its the adhesive activity and signaling role during fetal liver development.

Materials and methods

Mice. C57BL/6J mice were purchased from CLEA Japan. *AML1* mutant mice were generated as described previously [13].

Antibodies. Anti-Liv2 and anti-Liv8 antibodies were prepared and purified as described [9]. Anti-Liv8 antibody was biotinylated by using EZ link Sulfo-NHS-Biotin (Pierce) and anti-Liv2 antibody was labeled with Alexa Fluor 488 by using Zenon labeling kit (Invitrogen). Fluorescein isothiocyanate (FITC)-conjugated and unconjugated anti-CD44 (IM7 and KM114), FITC-conjugated anti-CD71 (C2) and phycoerythrin (PE)-conjugated anti-TER-119 antibodies were purchased from Becton Dickinson Biosciences, anti-Myc (9E10) and anti-FLAG (M2) antibodies were from Sigma, anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 6C5) antibody was from CHEMICON.

Immunohistochemistry. Paraffin-embedded and frozen sections were immunostained according to previously described protocols [9].

Cell culture and transfection. LO cells were established as described previously [14], and maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 15% fetal calf serum (FCS), 10 ng/mL mouse OSM (Sigma) and antibiotics. cDNA encoding CD44 tagged with FLAG at C-terminal (FLAG-CD44) were cloned into mammalian expression vector pCMV5. For gene expression analysis, 293T cells were plated and transfected 1 day later with 5 μg of plasmid DNA using LipofectAMINE 2000 (Invitrogen).

Immunoblotting. Fetal liver (E11.5), LO and COS-7 cells were washed and homogenized in phosphate-buffered saline (PBS). The homogenates were centrifuged at 900*g* for 10 min at 4 °C, and the supernatants were centrifuged at 100,000*g* for 30 min at 4 °C. The resulting membrane pellets were used as membrane fractions. For the deglycosylation of membrane fraction, samples were incubated with PNGase F (PROzyme) or neuraminidase (nacalai tesque) according to the manufacturer's instructions. Immunoprecipitation and immunoblotting were performed as described [15].

Purification of Liv8-antigen and mass spectrometry. LO membrane fraction was lysed in 50 mM Tris–HCl (pH 7.5), 1% Lubrol PX (lysis buffer), and centrifuged at 100,000*g* at 4 °C for 1 h. The supernatant was applied on WGA agarose column (Seikagaku Corporation) equilibrated with lysis buffer. The column was washed with 1 mM phosphate buffer (pH 7.4), 1 M NaCl, 0.1% sodium-cholate (wash buffer), and the protein was eluted with wash buffer containing 250 mM N-acetyl-D-glucosamine (GlcNAc). The GlcNAc eluant was then applied on Phenyl-Sepharose column (GE healthcare) equilibrated with wash buffer, and the protein was eluted with a liner gradient of 1000–0 mM NaCl and 0.1–2% of sodium-cholate. The Liv8-positive fractions were adjusted to 1 mM $CaCl_2$, then applied on the hydroxyapatite Bio-Gel HTP Gel column (Bio-Rad) equilibrated with 1 mM phosphate buffer, 0.3 mM $CaCl_2$, 0.1 % Lubrol PX. The protein was eluted with a liner gradient of 1–400 mM phosphate containing 0.1% Lubrol PX and 0.1 mM EDTA. The Liv8-positive fractions were diluted with 50 mM Tris–HCl (pH 7.5), 0.1% Lubrol PX (dilution buffer) and applied on Mono Q HR5/5 column (GE healthcare) equilibrated with dilution buffer. The protein was eluted with a liner gradient of 0–1 M NaCl in dilution buffer. Purified Liv8-antigen was concentrated with VIVASPIN (VIVASCIENCE) and separated by SDS–PAGE. The proteins were visualized with Silver Stain II Kit Wako or negative gel stain MS kit according the manufacturer's instructions (Wako). The appropriate position was cut out and washed with 5% acetate, 50% methanol, and dehydrated with 66% acetonitrile, 17 mM NH_4HCO_3. The protein was reduced by 10 mM dithiothreitol, alkylated by 55 mM iodoacetamide, and digested by trypsin. The peptide solution was placed on the target tip with α-cyano-4-hydroxy cinnamic acid as the matrix. The spectra of the peptides were obtained using Ultraflex mass spectrometer (BRUCKER) and Voyager DE-STR (Applied Biosystems).

Fluorescence-activated cell sorter (FACS) analysis. Freshly isolated fetal livers were incubated with Liver Perfusion Medium and Liver Digestion Medium (Invitrogen) at 37 °C. The cells were dissociated by pipetting, washed with PBS, and reacted with fluorescein-labeled antibodies according to previously described protocol [11]. The labeled cells were analyzed using FACSCalibur (Becton Dickinson Biosciences).

Results

Expression of Liv8 in murine fetal liver

As almost all cells were Liv8$^+$ in fetal liver at E11.5 (Fig. 1A), we first examined the expression of Liv8 in hepatoblasts, and found that Liv8 is expressed in Liv2$^+$ hepatoblasts in fetal liver at E11.5 (Fig. 1B; arrowheads). Next, we examined the expression of Liv8 in fetal livers during E9.5 to E13.5. Interestingly, Liv8 was not expressed in hepatoblasts at E9.5 (Fig. 1C, dotted line). The ratios of Liv8$^+$ cells in fetal livers transiently increased and reached its maximum at E11.5, and then decreased at E12.5. Thus, hepatoblasts were Liv8$^-$ at E9.5 and became Liv8$^+$ at E11.5.

Previously, we reported that hematopoietic progenitor cells in fetal liver are Liv8$^+$ at E11.5 [11]. So, we next investigated the expression of Liv8 in AGM region, in which adult-type definitive hematopoiesis begins (Supplementary Fig. S1). Endothelial cells in AGM region at E9.5 were Liv8$^+$, and putative hematopoietic cells protruding from dorsal aorta at E11.5 were also Liv8$^+$. These results indicate that hematopoietic progenitor cells are Liv8$^+$ and hepatoblasts change from Liv8$^-$ at E9.5 to Liv8$^+$ at E11.5 in developing fetal livers.

Identification of Liv8 as CD44

To identify Liv8, we screened Liv8$^+$ cell line and found an endothelial-like cell line, LO cell, established from AGM region [14]. A band of molecular weight of 90-kDa recognized by anti-Liv8 antibody was detected in murine fetal liver and LO cells but not COS-7 cells (Fig. 2A). The band was disappeared by incubation with PNGase F that cleaves asparagine-linked oligosaccharides from glycoprotein, and was shifted to low molecular weight side by incubation with sialidase (Supplementary Fig. S2). These results indicate that Liv8-antigen is a 90-kDa glycoprotein, so we screened Liv8-antigen binding lectins and found Liv8-antigen bind to wheat germ agglutinin (WGA), a lectin that binds to hybrid type asparagines-linked oligosaccharides (data not shown). We purified Liv8-antigen from LO cells by various columns including WGA column, using anti-Liv8 blot as an index (see Materials and methods). Purified Liv8 was subjected to mass analysis. The amino acid sequence of