Glycoconj J (2009) 26:1213–1223
DOI 10.1007/s10719-009-9240-2

High levels of E_4-PHA-reactive oligosaccharides: potential as marker for cells with characteristics of hepatic progenitor cells

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Received: 18 September 2008 / Revised: 23 March 2009 / Accepted: 14 April 2009 / Published online: 15 May 2009
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Abstract Oligosaccharides serve as markers of the cell surface and have been used as certain kinds of tumor markers. In the present study, we established a simple method for isolating hepatic progenitor cells using a lectin, which recognizes a characteristic oligosaccharide structure. Rat liver epithelial (RLE) cells, which have been established as a hepatic stem-like cell, were used to identify characteristic oligosaccharide structures on hepatic stem cells. As a result from lectin micro array, several types of lectin including E4-PHA were identified to bind RLE cells specifically. Furthermore, lectin blot and lectin flow cytometry analyses showed that binding to E_4-PHA lectin was significantly increased in RLE cells, compared to hepatocytes, and hepatoma cells. The induction of differentiation into a hepatocyte lineage of RLE cells by treatment with Oncostatin M and dexamethasone resulted in a decrease in E_4-PHA binding. Using an E_4-PHA column, we succeeded in isolating hepatic stem cells from LEC (Long-Evans with cinnamon coat color) rat livers with fluminant hepatitis. The characteristics of the established cells were similar to RLE cells and had a potential of proliferating in rat liver. These results suggest that oligosaccharides can serve as a novel marker for the isolation of the hepatic progenitor cells.

Keywords Hepatic stem cells · Lectin · E4-PHA · Liver · Lectin micro array

Abbreviations

RLE cell	rat liver epithelial cell
E_4-PHA	Erythroagglutinating phytohemagglutinin
LCA	Lens culinaris agglutinin
WGA	Wheat germ agglutinin
LEC rats	Long-Evans with cinnamon coat color rats
GFP	Green Fluorescence Protein
LEC-HP cells	LEC rat-derived hepatic progenitor cells

Introduction

Hepatic progenitor cells have received great attention as potential candidates for liver-directed therapy and as a tool

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for regenerative medicine prior to liver implantation. The development of the liver is complicated and hepatic stem cells could exist in certain organs including the liver, bone marrow, and the fetal cord vein [1]. It has also been reported that hematopoietic cells can fuse with hepatocytes in an injured liver [2]. To purify hepatic progenitor cells from these organs rapidly, a simple marker is needed, because complicated isolation procedures would decrease their efficiency and damage cells. Oligosaccharide structures are changed during differentiation, birth, and carcinogenesis, and a reactive set of glycosyltransferases is involved in the synthesis of each oligosaccharide [3]. These glycosyltransferases are expressed in an organ-specific or cell-specific manner, and therefore, oligosaccharides could serve as a surface marker of specific cells. For example, both *N*-acetylglucosaminyltransferases III (GnT-III) and -V (GnT-V), which are involved in the synthesis of bisecting GlcNAc or β1–6 branching in -glycans, are expressed at very low levels in normal liver, but are up-regulated in chronic liver disease, fetal liver and liver regeneration in animal models [4, 5]. If hepatic progenitor cells appear during these processes, an enhancement in GnT-III and GnT-V expression could in part alter the oligosaccharide structures on such kinds of special cells in the liver. To isolate hepatic progenitor cells from certain tissues such as an injured liver, fetal liver, and bone marrow using oligosaccharide markers, a characteristic oligosaccharide structure for hepatic progenitor cells needs to be determined.

RLE (rat liver epithelial) cells, hepatic stem-like cell lines, were previously established from normal rat liver [6]. Until recently, a variety of studies in terms of RLE cells and hepatic differentiation have been reported [7, 8]. RLE cells can be cultured for up to 33~35 passages, maintaining their hepatic stem cell-like characteristics and are differentiated into hepatocytes when they are transplanted into the rat liver. In the present study, we analyzed oligosaccharide structures of RLE cells compared to hepatocytes, non-parenchymal hepatic cells, and hepatoma cell lines, using several types of lectins. We found that high levels of binding to E4-PHA were observed in RLE cells and decreased binding was one of the signs for RLE cell differentiation into hepatocytes. A detailed analysis of the oligosaccharide structures of RLE cells was also performed by mass spectrometry.

We also isolated hepatic progenitor cells from LEC rat (Long-Evans cinnamon coat color rat) livers with fluminant hepatitis using an affinity column with the E_4-PHA lectin. LEC rats carry a defect in the *ATP7B* gene, resulting in the accumulation of copper in their livers and are used as a model rat for human Wilson's disease [9]. They spontaneously develop fluminant/acute hepatitis at approximately 20 weeks of age after birth and the surviving rats subsequently develops chronic hepatitis followed by liver cancer. It has been reported that hepatic progenitor cells appeared in the LEC rat liver at the stage of acute/chronic hepatitis [10]. Established hepatic progenitor cells from LEC rat livers (LEC-HP cells) with fluminant hepatitis, isolated using the E_4-PHA lectin were characterized in terms of oligosaccharide structures, gene expression, and potential for differentiation into hepatocytes.

Materials and methods

Cell culture

RLE cells in 19 passages were kindly provided by Dr Snorri S Thorgeirsson (National Cancer Institute/National Institutes Health), and detailed information for RLE cells were described previously [6]. RLE cells in 19~24 passages were used in this experiment. RLE cells were cultured in HAM'S F-12 medium (ICN biomedicals, inc, Irvine, CA) containing 10% fetal bovine serum (Sigma, Saint Louis), 50 μg/ml gentamicin (Wako, Osaka, Japan). Cells were made for passage weekly and used between passages 24 and 32 in this study. Rat hepatocytes and non-parenchymal hepatic cells were prepared from 8 week-old Sprague Dawley rats (body weight approximately 200 g, Charles Liver, Japan) using a two-step collagenase perfusion methods described by Selgen [11]. These cells were separated by centrifugation and cultured in DMEM (Sigma) containing 100 μg/ml kanamycin (Sigma), 10^{-5}M dexamethasone (Wako), 10^{-7} M insulin and 10% FBS (fetal bovine serum). The medium was changed at 3 h and 24 h after plating. After 48 h, cells were collected and used in lectin blot or lectin flow cytometry analyses. A rat hepatoma cell line, AH66 [12] was donated by Japanese Cancer Research Resources Bank and cultured in RPMI-1640 medium (Sigma) containing 50 μg/ml streptomycin, 50 units/ml penicillin (ICN biomedicals, inc) and 10% FBS. Under subconfluent conditions, RLE cells and AH66 cells were collected and used in lectin blot or lectin flow cytometry analyses.

Lectin micro array

Total pattern of oligosaccharide structures in hepatocytes, RLE cells and hepatoma cells were investigated with evanescent-field fluorescence–assisted lectin micro array [13]. 43 kinds of lectin were immobilized on the glass slide with triplicate, and approximately 250 ng/ml of cellular proteins in phosphate-buffered saline with 1% triton X-100 were applied to the array. To label target glycoproteins, Cy3 monoreactive dye (GE Healthcare Biosciences, the Chalfont St Giles, UK) was used in this analysis. Detail procedures were described previously [13]. Fluorescence

intensity of all lectins and that of lower signal-lectins were analyzed independently.

Lectin blot and lectin flow cytometry analyses

For lectin blot analyses, cells, plated on 10-cm dishes, were washed twice with PBS and scraped into 1 ml of PBS, and then centrifuged at 3,000 rpm for 5 min. The pellets were homogenized in TNE buffer, which contained 10 mM Tris-HCl (pH 7.8), 1% NP-40, 0.15 M NaCl, 1 mM EDTA and a mixture of protease inhibitors (WAKO) for 20 min on ice, and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant fraction was used in this assay. Protein concentrations were determined with a BCA kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. For lectin blot analyses, 20 μg of proteins extracted from the cells were electrophoresed on a 10% polyacrylamide gel and then transferred onto a nitrocellulose membrane. After blocking with PBS containing 3% BSA overnight at 4°C, the membrane was incubated with 1 μg/ml of various biotinylated lectins (Seikagaku Corp., Tokyo, Japan) for 1 h. The washing and developing procedures have been described previously [5]. Reactive glycoprotein bands were visualized by chemiluminescence, using an ECL system (GE Healthcare Biosciences). For lectin flow cytometry, cells on 10-cm dishes were treated with trypsin containing 0.2% EDTA and centrifuged at 1,000 rpm for 5 min after the addition of a three fold volume of DMEM containing 10% FBS. Cell pellets were suspended in 100 μl of PBS containing 0.1% BSA. Fluorescein isothiocyanate-labeled lectins, including E_4-PHA, WGA and LCA were added to a final concentration of 5 μg/ml. After staining for 20 min on ice, the cells were pelleted and washed 3 times with cold PBS, followed by sorting on a fluorescence activated cell sorter (FACS-SORT, Becton Dickinson, Mountain View, CA). Establishing gates to monitor live cells only, but not cell debris eliminated the background. Unstained cells served as controls. A fluorescence histogram and mean fluorescence were determined and analyzed from these data using the Macintosh Cell Quest computer program (FACS-SORT). Binding capacity to lectins was evaluated as the difference between the mean fluorescence of stained cells and the mean auto fluorescence of the cells.

In vitro differentiation of RLE cells to hepatocyte lineage

In vitro differentiation of RLE cells to hepatocytes was induced as described previously [8]. The following inducing factors were added to the medium in this sequence. After treatment with 1 μM 5-Aza-2'-deoxycitidine (Sigma) for 2 days, RLE cells were cultured in a 10% serum DMEM/HAM'S F-12 (1:1) medium containing 10 ng/ml Oncostatin M (OSM) (R&D Systems, Minneapolis, MN), 10 mM nicotinamide (Wako), 2 mM L-glutamine (ICN biomedicals, inc), 10^{-6} M dexamethasone and 1 μg/ml insulin. Control cells were cultured in standard HAM'S F-12 medium with 10% FBS for the same period of time. After 7 days, we performed a flow cytometry analysis using the E_4-PHA lectin and an RT-PCR analysis of hepatic differentiation markers.

Reverse transcription-polymerase chain reaction analysis

Total cellular RNA was prepared from cells on culture dishes using TRIzol Reagent (Invitrogen, Carlsbad, CA). Five μg of total RNA were reverse transcribed into complementary DNA, using the SuperScript First-Strand cDNA Synthesis System (Invitogen) with oligo (dT) as a primer. Complementary DNA was used as a template for amplification of hepatic markers in a PCR reaction. Primer sequences and PCR conditions are summarized in Table 1. β-actin was amplified as an internal control. The PCR

Table 1 Primer sequences and PCR conditions

Gene	Primer sequence (5′–3′)		Annealing temperature (°C)	PCR cycles	Product size (bp)
albumin	F	GAGAAGGTCACCAAGTGCTGTAGT	65	38	147
	R	CTGGGAGTGTGCAGATATCAGAGT			
C×32	F	TAACAGCGTCTGCTATGACC	65	38	336
	R	GAAGGCCTCACACTTGACCA			
TAT	F	CTATCACGACACGTTAAGCT	60	36	282
	R	ACAAGCCTCCAGCATCATCA			
CK19	F	ACCATGCAGAACCTGAACGAT	65	38	87
	R	CACCTCCAGCTCGCCATTAG			
AFP	F	CTGTCACTGCTGATTTCTCTGG	65	38	152
	R	GTCCTTTCTTCCTCCTGGAGAT			
β-actin	F	GAAGATTTGGCACCACAGTTT	55	25	595
	R	TTGAATGTAGTTTCATGGAT			

products were electrophoresed on 2% agarose gels and visualized using ethidium bromide

Mass spectrometry

N-linked oligosaccharides on RLE cells and hepatocytes were liberated by treatment with PNGase F (Takara bio corp. Shiga, Japan) and then labeled with 2-aminopyridine, using a Glyco-Tag reagent kit. Excess reagent was removed by gel filtration and the resulting PA oligosaccharides were analyzed by mass-spectrometry. Mass measurements were carried out using a matrix-assisted laser-desorption/ionization (MALDI) quadruple ion trap time-of flight mass spectrometer (AXIMA-QIT, Shimadzu). Details of this procedure have been described previously [14].

Establishment of hepatic progenitor cells from LEC rat liver

Liver cells were prepared from a 23 week-old LEC rat by the two-step collagenase perfusion method as described above. After the collagenase perfusion, the liver cells were centrifuged at 50*g* for 5 min four times. The pellet was suspended in 2 mM EDTA/PBS containing 0.5% BSA, and biotinylated E_4-PHA lectin was added to a final concentration of 10 μg/ml. After incubation at 4°C for 5 min, the cells were washed twice with cold 2 mM EDTA/PBS. The cells were resuspended in 2 mM EDTA/PBS and incubated with anti-biotin micro beads at 4°C for 20 min. After washing twice with 2 mM EDTA/PBS, cell separation was performed by auto MACS (Magnetic Cell Sorting and Separation of Biomolecules, Miltenyi Biotec, Bergishu Gladbach, Germany). Both positive (bound to E_4-PHA) and negative (unbound to E_4-PHA) fractions were collected and cultured on collagen-coated dishes with D-MEM containing 10% FBS, 10^{-5}M dexamethasone, 10^{-7}M insulin and 100 μg/ml kanamycin. Medium was replaced at 3 h and 24 h after plating, and thereafter the culture medium was changed every three days. Two weeks after plating, a small number of colonies appeared on the dishes derived from the E_4-PHA positive fraction. Each colony was then picked-up after treatment with trypsin, and cultured in HAM'S F-12 medium containing 10% FBS, 50 μg/ml kanamycin and 50 units/ml penicillin. After the cells formed a confluent monolayer in 10 pieces of 10 cm dishes, they were treated with trypsin and stocked in liquid nitrogen. These cells were defined as 5 passage cells and cells less than 20 passage were used for the experiment. These cells were denoted as LEC rat-derived hepatic progenitor cells (LEC-HP cells).

For differentiation studies, isolated cells were plated on a fibronectin-coated dish and cultured in DMEM/HAM'S F-12 (1:1) medium containing 10% FBS, 50 ng/ml hepatocyte growth factor (HGF), 10 mM nicotinamide, 2 mM L-glutamine, 10^{-6}M dexamethasone and 1 μg/ml insulin for 7 days. Total RNA was extracted from these cells and RT-PCR analysis of hepatic markers was performed as described above.

Cell transplantation

LEC-HP cells in sub-confluent conditions were treated with trypsin and resuspended in fresh medium containing 10% FBS. The cell suspension was centrifuged at 1,000 rpm for 5 minute. The cell pellet was rinsed with PBS twice and the density of the cells was adjusted to 5 x 10^7 cells/ml with PBS. Twenty-four hours before cell transplantation, female 6-week-old Sprague Dawley rats (body weight approximately 110 g) were treated with an intraperitoneal injection of 2 ml/kg body weight of carbon tetrachloride in a 1:1 (vol/vol) diluted in olive oil. After 1 day, 5x10^6 LEC-HP cells in 0.1 ml of PBS were injected into the livers of these rats. The cells were transfected with a GFP (green fluorescence protein) expression vector (pEGFP-N1, Clonetech, CA, USA). A positive clone of GFP expression in LEC-HP cells was used in this experiment. The rats were sacrificed under anesthesia 4 weeks after cell transplantation and their livers were fixed in 4% paraformaldehyde for histological analysis. Immunohistochemical analysis for GFP staining was performed according to the methods as reported previously [15]. Liver tissues fixed in 4% paraformaldehyde were embedded in paraffin. A mouse anti-GFP antibody (Chemicon, Temecula, CA) was used at a dilution of 1:500. HRP-conjugated anti-mouse antibody (Promega, CA) was used as the second antibody and positive staining was visualized using diaminobenzidine (DAKO, Carpinteria, CA). Counterstaining was performed with hematoxylin.

Results

Lectin micro array

Lectin micro array showed slight increases / decreases in binding to several kinds of lectins among hepatocytes, RLE cells and rat hepatoma cell lines, AH66 (Fig. 1a). Low binding to L4-PHA in these cells would be due to the character of this lectin although GnT-V and β1–6 GlcNAc structures are increased in cancer cells and liver regeneration [4, 5]. No specific changes for RLE cells were observed in cases of lectins, which showed higher signals (data not shown). However, there were significant changes in binding for RLE cells in cases of lower signal lectins (Fig. 1b). Most prominent changes of lectin binding in RLE cells were observed in the case of E_4-PHA and RCA-I. Detail characters of each lectin for distinct oligosaccharides were described previously by Rudiger and Gabius [16].

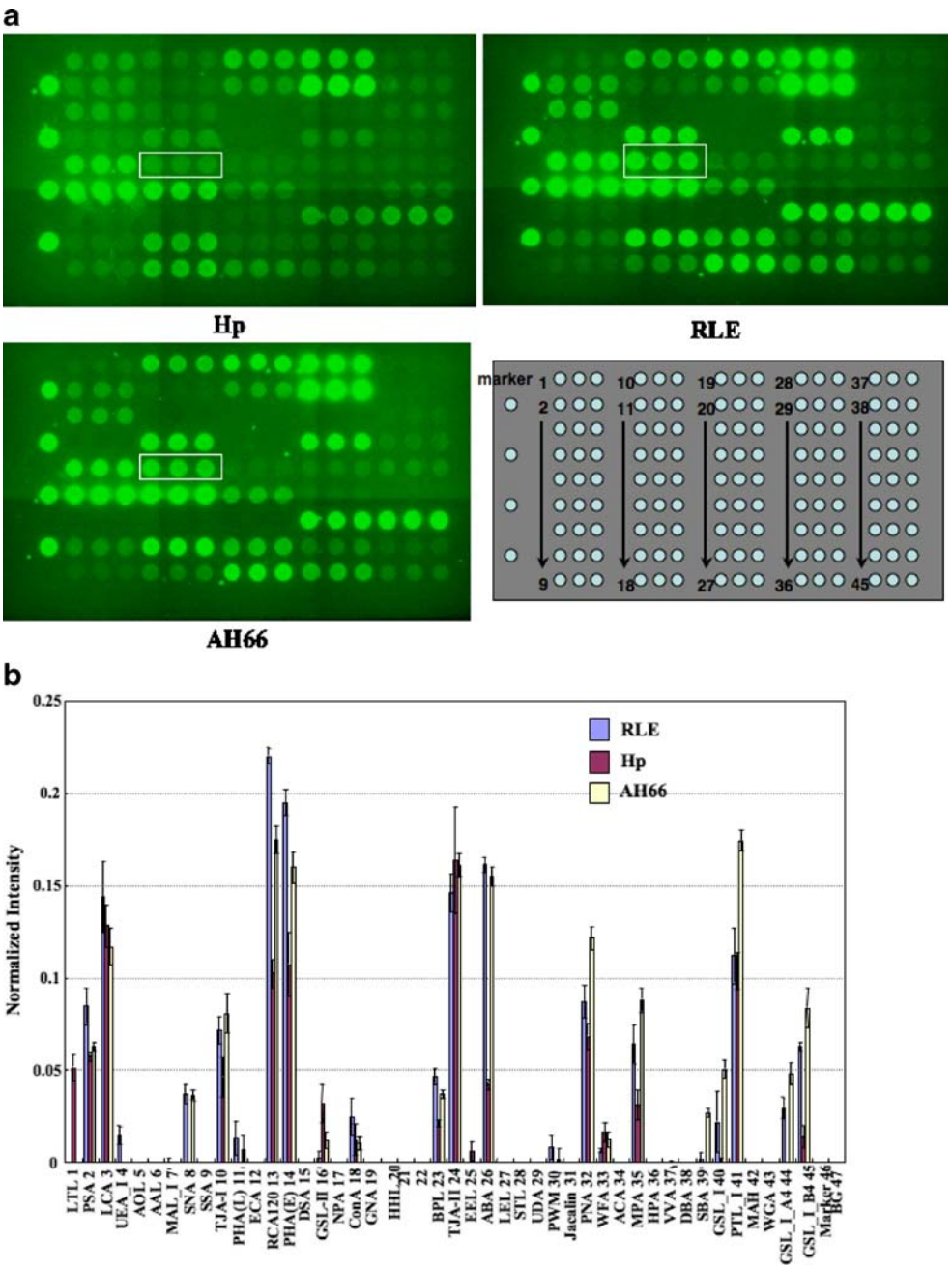

Fig. 1 Lectin micro array a 1 μg of cellular proteins derived from hepatocytes (Hp), RLE cells, and AH66 cells were labeled with Cy3. Lectin micro array analysis was performed as described in Materials and Methods. Each lectin was attached on the dish at triplicate. The number in the right-lower panel was consistent with the lectin number on Figs. 1(B). A white square indicates the data of E_4-PHA. **b** Fluorescence levels of only lower signal-lectins were automatically determined and plotted with SD of 3 time experiments

These data should be confirmed with other methods such as lectin blotting or flow cytometry.

RLE cells have high affinity for E_4-PHA lectin

To analyze the expression pattern of oligosaccharides on RLE cells in details, compared with hepatocytes, nonparenchymal cells or rat hepatoma cells, AH66, we performed western blotting using 8 different lectins. Representative data for 3 lectin blots such as E_4-PHA, LCA and WGA was shown in Fig. 2a. E_4-PHA binds to a bisecting GlcNAc, which is a product of GnT-III [17], LCA binds to α1–6 fucosylated *N*-glycans [18] and WGA binds to GlcNAc, or sialylated glycans or hybrid type *N*-glycans [19]. CBB staining of the 4 types of cells showed that the total proteins of RLE cells were similar to those of nonparenchymal cells and different from those of hepatocytes or hepatoma cells. This result was consistent with the data of lectin micro array. In contrast, binding to the E_4-PHA lectin was dramatically enhanced in RLE cells, compared to other types of cells. In the LCA lectin blot, a high affinity of binding was observed in nonparenchymal cells, while it was not observed in RLE cells. No dramatic differences in affinity to other lectins including WGA were observed (data

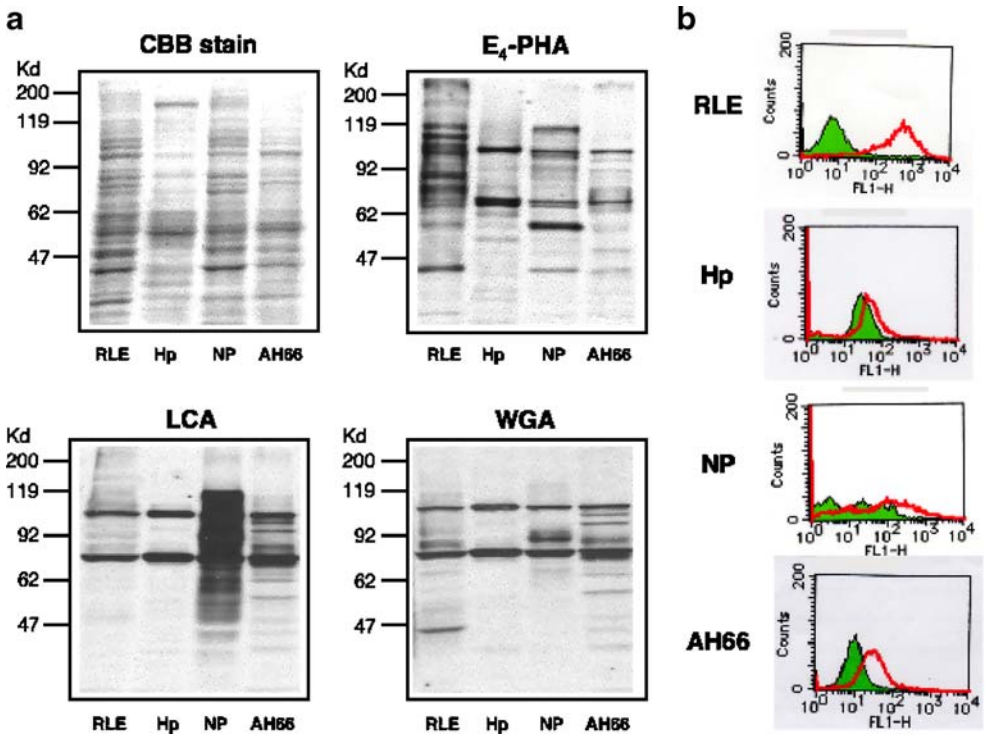

Fig. 2 Analyses of oligosaccharide structures by lectins a Analysis of oligosaccharide structures by Western blot using several types of lectins. 20 μg of cellular proteins from RLE, hepatocytes (Hp), non-parenchymal cells (NP), and AH66 were electrophoresed on 10% SDS-PAGE. After blotting onto a nitrocellulose membrane, a western blot analysis using several types of lectins and CBB staining were performed. **b** Analysis of oligosaccharide structures on cell surface by lectin flow cytometry using E_4-PHA. Binding to E_4-PHA lectin was dramatically increased in RLE cells. Detail procedure was described in Materials and Methods

not shown). The oligosaccharide structures of cell surface proteins were next analyzed by lectin flow cytometry using E_4-PHA. As shown in Fig. 2b, a marked increase in binding to E_4-PHA was observed in RLE cells. In the case of nonparenchymal cells, at least 3 types of cellular populations binding to E_4-PHA were observed, suggesting that cell population is heterogeneous. Hepatic progenitor cells such as RLE cells could exist in this cell population. Collectively, the staining pattern of E_4-PHA lectin blot and flow cytometry of RLE cells was significantly different from the other cells, suggesting that cell surface oligosaccharides could be a good marker for identifying hepatic stem-like cells.

Mass spectrometry analysis on oligosaccharide structures of RLE cells

We investigated the oligosaccharide structures of RLE cells and hepatocytes in detail. Representative MS^n analytical data are shown in Fig. 3. Each oligosaccharide structure was identified by a similarity search in the MS^n spectral library for signal intensity profiles obtained from MS^3 spectra of fraction A, B and M5~M9. There were no differences between RLE and hepatocytes in terms of the high mannose structures identified by the MS^3 spectra of fractions M5~M9, but peak M5 was decreased in RLE cells. Peaks A and B

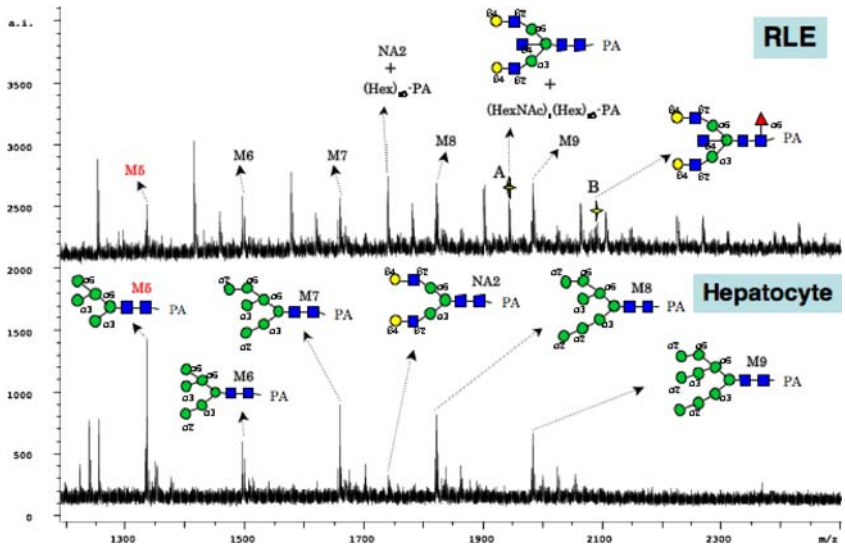

Fig. 3 Analyses of oligosaccharide structures by mass spectrometry Whole oligosaccharides derived from RLE cells and hepatocytes were labeled with 2-amino-pyridine followed by mass-spectrometry (MS) analysis. MS/MS analysis on peaks A and B showed that these oligosaccharides contained the bisecting GlcNAc structure, which was recognized by E_4-PHA

were detected only in RLE cells, and the structures obtained from these fractions were estimated to be diantennary *N*-glycans having bisecting GlcNAc by MS^3 analysis. Diantennary *N*-glycans with a bisecting GlcNAc are the most suitable oligosaccharide structures, recognized by E_4-PHA. While the structure of NA2 was identified in both RLE and hepatocytes, undefined structures such as PA-labeled hexose oligomers or *N*-acethylhexose linked hexose oligomers were detected only in RLE cells.

Changes in oligosaccharide structures during differentiation of RLE cells

Both lectin blots and lectin FACS analyses revealed that E_4-PHA binding was increased in RLE cells. We then investigated whether or not E_4-PHA binding to RLE cells changed during their differentiation into hepatocytes. RLE cells were cultured in medium containing 10 ng/ml OSM and 10^{-6}M dexamethasone for seven days. As shown in Fig. 4a, a remarkable enlargement in cell size and a reduction in the nucleus and cytoplasm ratio were observed. Moreover, RT-PCR analysis revealed an induction in the expression of hepatocyte specific markers such as albumin, connexin 32 (Cx32) and tyrosine aminotransferase (TAT) (Fig. 4b), indicating that the RLE cells had differentiated into a hepatocyte lineage. In these differentiated RLE cells, binding to E_4-PHA was markedly decreased in flow cytometry (Fig. 4c) and slightly decreased in lectin blotting (Fig. 4d). Basal staining in flow cytometry of original and differentiated RLE cells was different because morphology of these 2 cells were dramatically different as shown in Fig. 4a. These data suggest that the oligosaccharide structures on the cell surface of RLE cells changed during differentiation and strong E_4-PHA binding is a characteristic of an immature phenotype of RLE cells.

Isolation of E_4-PHA-positive cells from rat liver

To demonstrate that the E_4-PHA lectin can be used in the isolation of hepatic stem-like cells, we obtained hepatic progenitor cells from LEC rat livers using an affinity column with the E_4-PHA lectin. We used a 23 week-old LEC female rat, which suffered from fluminant hepatitis. Liver cells prepared from LEC rats through a collagenase-perfusion method were separated into E_4-PHA positive and negative fractions by auto MACS as described in Materials and Methods. The cells prepared from each fraction were plated onto collagen-coated culture dishes, and then cultured for 14 days. As shown in Fig. 5a-2, most of the hepatocytes in the E_4-PHA-negative fraction were dead after 14 days in culture. In contrast, the E_4-PHA positive fraction consisted of various types of cells, and most of

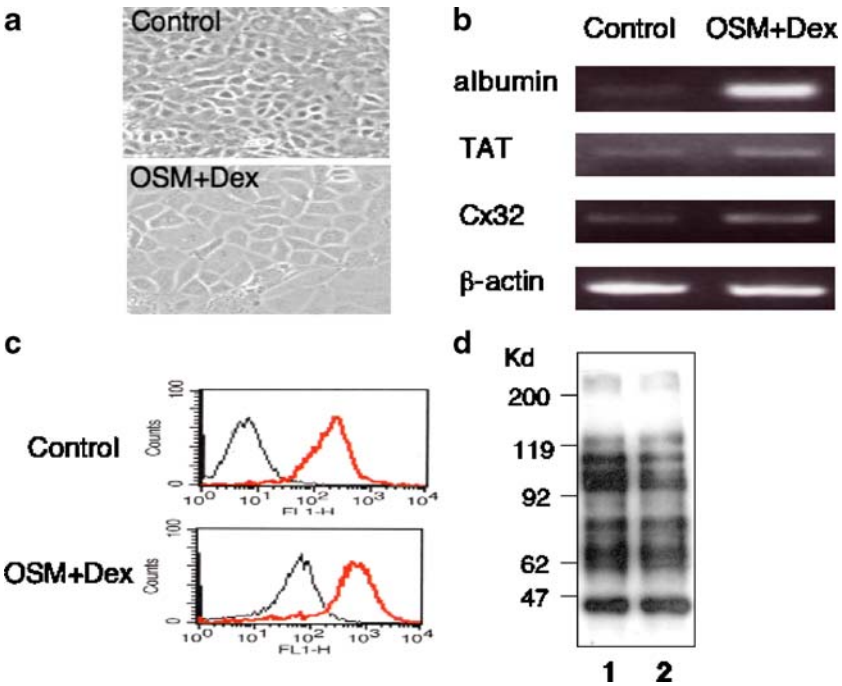

Fig. 4 Differentiation of RLE cells and changes in oligosaccharide structure **a** Morphological changes of RLE cells treated with oncostatin M and dexamethasone. **b** Total RNA was extracted from RLE cells treated with/without oncostatin M and dexamethasone for 7 days. RT-PCR analysis of hepatocyte-related genes such as albumin, TAT, and Cx32 was performed. β–actin was used as a control. **c** Oligosaccharide structure of RLE cells treated with/without oncostatin M and dexamethasone for 7 days were analyzed by flow cytometry using E_4-PHA. **d** The same cells were analyzed by lectin blotting, using E4-PHA. Lane 1 indicates control cells and lane 2 indicates cells treated with oncostatin M and dexamethasone

which could not attach to the dishes. The cells attached to the dishes in the E_4-PHA-positive fraction were less than half the size of hepatocytes. After two weeks in culture, several colonies composed of small and uniformly shaped cells appeared on the dishes of the E_4-PHA-positive fraction (Fig. 5a-1) and were replated onto non-collagen-coated dishes. These cells, designated LEC-HP (LEC rat-derived hepatic progenitor) cells, proliferated within a week and formed a monolayer with a cobblestone appearance (Fig. 5a-3).

Characterization of LEC-HP cells

We confirmed that the LEC-HP cells had a high affinity for E_4-PHA lectin compared with hepatocytes and non-parenchymal cells, as well as RLE cells. Interestingly, the staining pattern of other lectin blots of LEC-HP cells was similar to that of RLE cells, while slightly lower binding to the LCA lectin was observed in LEC-HP cells, compared to RLE cells (Fig. 5b). Based on the morphological observation, the LEC-HP cells appeared to be slightly larger in size compared with the RLE cells, but they contained the same ovoid nuclei and had a high nucleus/cytoplasm ratio. Although these two cells showed slightly different morphologies, patterns of oligosaccharide structures that were expressed seemed to be similar between these cells.

To investigate the properties of LEC-HP cells, we examined the mRNA expression of hepatic markers by RT-PCR analysis (Fig. 5c). LEC-HP cells expressed low levels of cytokeratin19 (a marker of bile duct cells) and AFP (a marker of immature hepatocytes) as well as RLE cells. In contrast, the expression level of albumin mRNA was slightly higher in LEC-HP cells than in RLE cells. These data suggested that LEC-HP cells are hepatic progenitor cells, similar to slightly differentiated RLE cells. We next demonstrated that LEC-HP cells could differentiate "*in vitro*". Culturing for seven days in medium containing 10 ng/ml HGF on a fibronectin-coated dish induced a high expression of hepatocyte markers such as albumin and Cx32 in LEC-HP cells (Fig. 5d). Therefore, LEC-HP cells could be considered to be hepatic progenitor cells with an immature phenotype, and have the potential to differentiate into hepatocyte lineage.

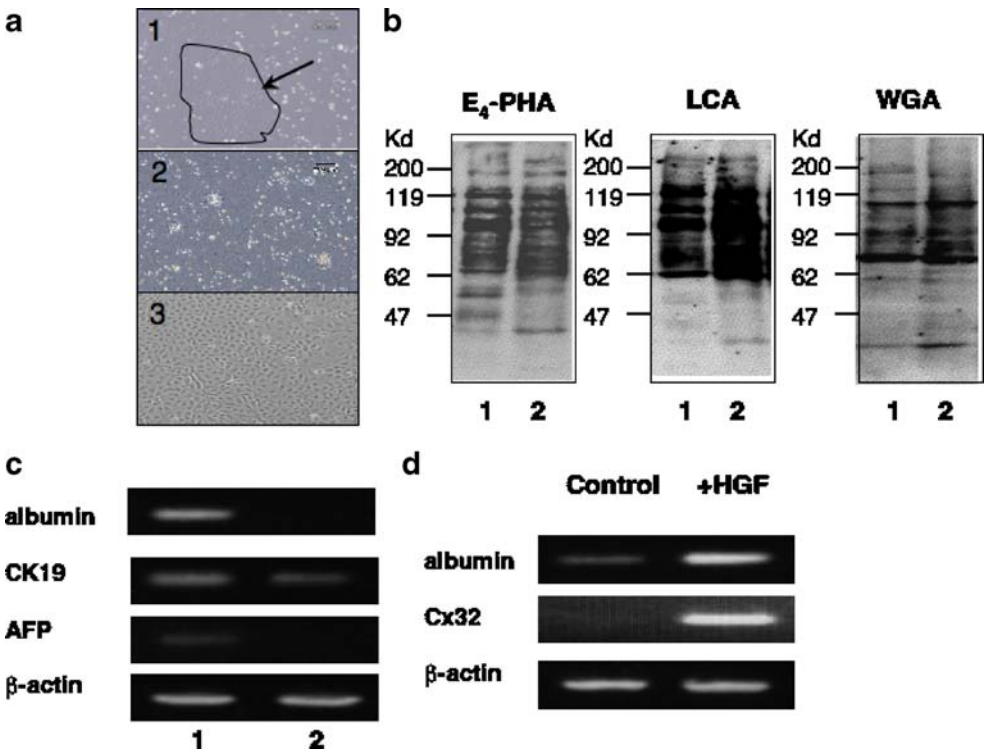

Fig. 5 Establishment of hepatic progenitor cells from LEC rat liver **a** Hepatic progenitor cells were isolated from LEC rat liver using an E_4-PHA lectin column, and then cultured for 14 days. Panel 1 or 2 indicates binding or non-binding cells to E_4-PHA, respectively. Most of cells binding to E_4-PHA column were dead because they were mature hepatocytes. An arrow in panel 1 indicates small colony of hepatic progenitor cells. Panel 3 indicates hepatic progenitor cells grown from cell population, which was bound to E_4-PHA. **b** Lectin blot analyses of total cellular proteins derived from LEC-HP (lane 1) and RLE (lane 2) cells. Detailed procedure can be found in Materials and Methods. **c** RT-PCR analyses of hepatic differentiation genes on LEC-HP cells (lane 1) and RLE cells (lane 2). **d** RT-PCR analysis on LEC-HP cells treated with HGF in fibronectin-coated dish. Expression of albumin and Connexin 32 mRNAs were investigated. β-actin was used as a control

LEC-HP cells can proliferate in rat liver

Since LEC-HP cells had a differentiation potential toward the hepatocyte lineage *in vitro*, we investigated whether or not they were transplantable in rat liver. To make better condition for proliferating of LEC-HP cells, rats were treated with CCl_4 before cell transplantation. As shown in Fig. 6, a small number of the cells were detected in the recipient rats liver, suggesting that LEC-HP cells could proliferate in the liver. No tumor formation was observed at 4 months after cell transplantation.

Discussion

In the present study, we identified a characteristic oligosaccharide structure for a hepatic stem-like cell, RLE compared to hepatocytes, non-parenchymal cells, and a hepatoma cell line. Lectin micro array showed increases in binding to E_4-PHA in RLE cells. Lectin blotting as well as lectin flow cytometry confirmed that binding to E_4-PHA lectin was specifically increased in RLE cells. When RLE cells were differentiated into hepatocytes by treatment with oncostatin M and dexamethasone, binding to E_4-PHA was decreased, suggesting that oligosaccharide changes were directly involved in the differentiation. This difference was more prominent in flow cytometry than lectin blotting, which would be dependent on kinds of lectin. In the case of E4-PHA lectin, flow cytometry is more sensitive than lectin blotting. As shown in Fig. 2a, E4-PHA blot showed similar band density in hepatocytes and AH66 cells, but stronger binding to E4-PHA was observed in AH66 cells, as judged by flow cytometry (Fig. 2b). In the procedure of differentiation of RLE cells, it is an interesting issue what percentage of RLE cells can be differentiated, while we have no answer to this question. Many passages of RLE cells resulted in a slight decrease of E4-PHA binding although it was dependent on several conditions. Many growth factors play a role in liver regeneration [20]. Recent studies showed that the bisecting GlcNAc residue on c-Met (hepatocyte growth factor receptor) enhances the HGF-induced biological response [21], and that bisecting GlcNAc also affects EGF signaling by modulating autophosphorylation or internalization of receptors [22, 23]. The sensitivity for these growth factors could be an important function for proliferation and differentiation of progenitor cells in the liver.

Changes of *N*-glycan structures on cell surface glycoproteins during differentiation have been observed in other types of cell lines [24, 25]. While it is unknown whether changes in binding to the E_4-PHA lectin is a cause of or a result of differentiation, the increase in E_4-PHA binding might indicate an immature phenotypic feature of RLE cells. An important question is raised here, *i.e.*, whether the increase in E_4-PHA binding observed in RLE cells is actually a specific marker for hepatic stem cells. To answer this question, we attempted to isolate hepatic progenitor cells, which preferentially bind to E_4-PHA, using a magnetic cell separation system. The fetal rat liver is a potentially rich source of bipotential progenitor cells for liver transplantation. There are many reports of the isolation of immature hepatic progenitor cells from fetal liver with a high-proliferative potential that can be differentiated into hepatocytes [26–28]. Therefore, we attempted to isolate hepatic progenitor cells from fetal rat livers or 10 day-old rat livers. However, E_4-PHA stimulated the agglutination of these cells. This phenomenon suggests two possibilities; one is that the bisecting GlcNAc structure is indeed abundant on the cell surface of immature hepatic cells in the fetal rat liver, the other is that immature hematopoietic cells also contain abundant bisecting GlcNAc structures. Because the fetal liver is an important organ of hematopoiesis in the fetus until development of the bone marrow, the fetal liver includes both hepatic and hematopoietic stem cells [29]. In fact, it has been reported that a human promyelocytic leukemia cell line HL-60 has bisecting GlcNAc residues on 1/2 of the cell surface N-glycans [26]. Therefore, an abundance of bisecting GlcNAc structures might be a common feature among hepatic and hematopoietic progenitor cells, as well as other distinguish-

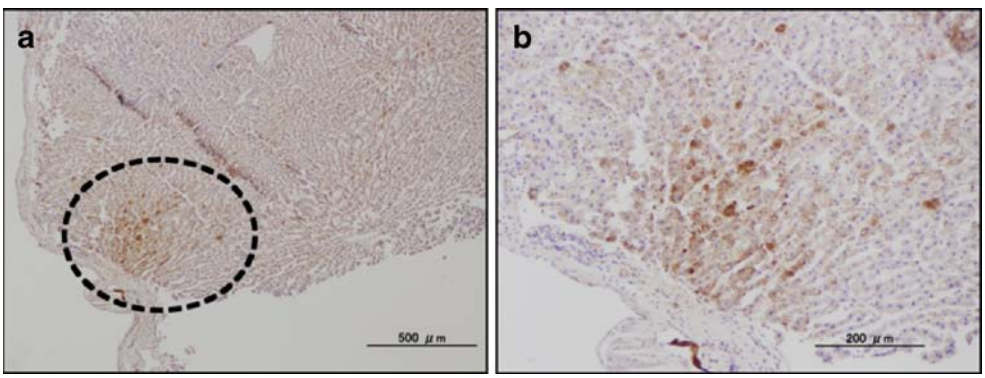

Fig. 6 Detection of proliferate foci of LEC-HP cells Approximately 5 x 10^6 LEC-HP cells labeled with GFP were directly transplanted into the liver with CCl_4-induced acute injury. After 1 month, proliferate foci of LEC-HP cells were detected with immunohistochemistry of GFP. Panel A or B indicates smaller or larger scale of the same section, respectively

able cell surface markers such as c-kit, CD34 and Thy-1, which are common to hematopoietic stem cells [30]. Functional implications of bisecting GlcNAc are reported previously [31, 32].

Hepatic stem cells could exist in the injured liver, although their origin might not be derived from the liver, but the bone marrow. Therefore, we attempted to isolate hepatic progenitor cells from LEC rat livers with fluminant hepatitis using an affinity column for the E_4-PHA lectin. While a variety of cells that bound to E_4-PHA column were isolated, most of the cells could not be made to passage. This is because some of the cells did not attach to the dish after a few passage and other cells had differentiated into hepatocytes after several passages. Established cells, designated as LEC-HP cells, expressed low levels of both hepatic and bile duct markers, and they were able to differentiate toward a hepatocyte lineage (Fig. 5d). However, LEC-HP cells were difficult to differentiate into bile duct cells as well as RLE cells. These data indicate that E_4-PHA as well as bisecting GlcNAc could be a marker for hepatic progenitor cells. Moreover, LEC-HP cells showed some similarities to RLE cells in lectin blot analysis (Fig. 5b), suggesting that oligosaccharide structures are common between both hepatic stem-like cell lines. When hepatic progenitor cells are used in cell therapy for severe liver diseases, methods for the isolation should be simpler, to maintain cell viability. If a mutated E_4-PHA lectin, which loses the ability of aggregation, exists, it would be an ideal tool for isolating hepatic progenitor cells. After establishing such kinds of lectin, we plan to isolate hepatic progenitor cells from umbilical cord blood by the same strategy as was used in this study. Although hepatic progenitor cells in the adult liver provide an ethically unacceptable source for clinical use, umbilical cord blood cells have advantages of native immune status and a relatively unshortened telomere length. While the E_4-PHA lectin might be suitable for the separation of hepatic progenitor cells in the umbilical cord vein, the marker should be re-investigated in terms of enhanced specificity for a hepatic lineage. An antibody that recognizes bisecting GlcNAc structures on glycoproteins has been established using mice lacking GnT-III [33], and it might be more useful for separating hepatic progenitor cells. In conclusion, we established a novel strategy for the isolation of hepatic progenitor cells using the E_4-PHA lectin and investigated their characters as hepatic progenitor cells. Further experiments would be required to know whether or not LEC-HP cells become cancer progenitor cells such as oval cells. If LEC-HP cells are not cancer progenitor cells, cell therapy would be possible.

Acknowledgement We would like to thank Dr. Snorri S Thorgeirsson (National Cancer Institute/National Institutes Health) for providing RLE cells. Japan Science and Technology Agency (JST) and the 21st Century Center of Excellence (COE) program and the Global COE program of Osaka University from the Ministry of Education, Culture, Sports, Science, and Technology of Japan supported this work, in part.

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