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Expression of CD44 in rat hepatic progenitor cells

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See Editorial, pages 1–4

***Background/Aims*: Small hepatocytes (SHs) are hepatic progenitor cells, but the phenotypical difference between SHs and mature hepatocytes (MHs) has never been demonstrated.**

***Methods*: The profile of gene expression was examined to clarify the difference between SHs and MHs by using a DNA microarray. Genes that were specifically expressed in SHs were identified and RT-PCR analysis of them was performed. Immunocytochemistry for CD44 standard form (CD44s) and variant form 6 (CD44v6) was performed using cultured SHs and the D-galactosamine (GalN)-injured rat liver. From the GalN-treated liver, CD44s$^+$ cells were obtained by sorting and RT-PCR analysis was performed.**

***Results*: Analysis using the DNA microarray and RT-PCR of them revealed restricted expression of CD44s and CD44v6 in SHs. In culture, CD44s appeared at day 3 and increased with the proliferation of SHs. CD44v6 expression was delayed compared to that of CD44s. With GalN-administration, CD44$^+$ hepatocytes appeared around periportal areas at days 3 and 4 and then decreased. Sorted CD44s$^+$ cells could form colonies and possessed hepatic markers.**

***Conclusions*: CD44 is a specific marker of SHs. The expression of CD44 mRNA and protein is restricted to SHs, and is up-regulated at the time when SHs start to proliferate both in vitro and in vivo.**

***Keywords*: CD44s; CD44v6; Small hepatocytes; Proliferation; Maturation**

1. Introduction

Small hepatocytes (SHs) are a subpopulation of hepatocytes that have high growth potential in culture [1–4]. The cells are less than half the size of mature hepatocytes (MHs), but they possess hepatic characteristics [5,6]. SHs can clonally proliferate to form colonies that survive for more than 5 months in defined medium [5,6] and can differentiate into MHs by interacting with hepatic nonparenchymal cells (NPCs) [7] or as a result of treatment with Engelbreth–Holm–Swarm gel [8]. Thus, we consider that SHs may be 'committed progenitor cells' that can further differentiate into MHs. Although SHs are primary cells that are freshly prepared from rat liver, they can also proliferate after cryopreservation [9].

The molecular mechanisms regulating the characteristics of SHs remain to be elucidated. In addition, their precise origin and location within the liver are not clear because the preparation of purified SHs is difficult and specific markers for SHs have never been identified. Therefore, it is important to identify specific genes and proteins expressed in SHs, especially cell membrane-integrated proteins, because it will be possible to clarify the characteristics of the cells by the methods of cell sorting.

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Abbreviations:: BECs, biliary epithelial cells; CD44s, CD44 standard form; CD44v, CD44 variant form; C/EBPα, CCAAT/enhancer binding protein α; CYP, cytochrome P450; GalN, D-galactosamine; HA, hyaluronic acid; LECs, liver epithelial cells; MH, mature hepatocyte; NPC, hepatic nonparenchymal cell; SH, small hepatocyte.

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The CD44 gene encodes for a family of alternatively spliced, multifunctional adhesion molecules that participate in lymphocyte–endothelial cell interactions as lymphocyte homing receptors [10–12], and in adhesion of cells to extracellular matrix [13], T cell activation and adherence [14], and metastasis formation [15]. CD44 standard form (CD44s) is composed of a short cytoplasmic tail, a transmembrane region and two extracellular domains. There are 10 additional exons (v1–v10). Although the expression of CD44 variant forms (CD44v) was initially considered to occur as a result of aberrant splicing in tumor cells, variant expression was subsequently detected in normal cells [16]. The expression of variant forms in hematopoietic cells has been reported [17–19].

In the present study, we found that both CD44s and CD44v6 were expressed in cultured SHs and their expression decreased with the maturation of the cells. Although biliary epithelial cells (BECs) also expressed CD44s, no other epithelial cells within the normal rat liver did. However, when the rat liver was severely injured by D-galactosamine (GalN) treatment, CD44s$^+$ epithelial cells appeared near Glisson's capsule in the liver lobules and, by using a specific antibody, the cells could sorted and thereafter cultured. These CD44s$^+$ cells expressed hepatic marker genes and could proliferate to form colonies consisting of SHs.

2. Materials and methods

2.1. Isolation and culture of SHs

Male F344 rats (Sankyo Lab Service Corporation, Inc., Tokyo, Japan) weighing 150–200 g were used to isolate hepatic cells by the two-step liver perfusion method of Seglen [20] with some modifications [2]. Briefly, suspensions of liver cells were centrifuged at 50×*g* for 1 min. The supernatant was used to prepare SHs and the precipitate was used to prepare MHs. The details of the isolation and culture procedure were previously reported [7]. After the number of viable cells was counted, cells were plated on dishes (7.5×10^4 cells/35-mm, 10×10^5 cells/100-mm dish; Corning Glass Works, Corning, NY). SH colonies cultured in a 100-mm dish were collected at day 14 and cryopreserved at −80 °C. The details of the method were previously reported [9]. After cryopreservation, SHs were thawed and suspended in the culture medium. To induce the maturation of SHs, they were overlaid with growth factor-reduced Matrigel® (BD Biosciences, Bedford, MA) at day 14 after thawing and the cells were then cultured. The details of the method were previously reported [8].

2.2. DNA microarray

Differences of the expression profiles of SHs and MHs were analyzed using a microarray approach. A DNA microarray spotted with 14,815 cDNAs (Agilent rat cDNA microarray kit) was purchased from Agilent Technologies, Inc. (Palo Alto, CA). Poly(A)$^+$ RNAs were prepared using ISOGEN (Nippon Gene, Tokyo, Japan) and mRNAs were prepared using a GenElute™-mRNA miniprep kit (Sigma Chem Co, St Louis, MO). Prepared mRNAs were labeled with Cy5- and Cy3-dUTP by reverse transcription. Analysis of the microarray was performed by Hokkaido System Science (Sapporo, Japan).

2.3. RT-PCR

Total RNA was isolated using ISOGEN. Reverse transcription and PCR amplification (RT-PCR) were performed in a one-step reaction according to the manufacturer's instructions (Invitrogen, San Diego, CA). Sequences of forward and reverse primers used are listed in Table 1. The constitutively expressed gene glycerol 3-phosphate dehydrogenase (GPDH) was also reverse-transcribed in a separate reaction as a qualitative and quantitative control.

2.4. Northern blot analysis

Northern blot analysis was performed as previously reported [21]. Probe labeling and RNA detection were performed according to the manufacturer's instructions for the AlkPhos Direct Labelling and Detection System with CDP-Star (Amersham Biosciences, Piscataway, NJ). For probes, the full-length CD44s and partial 450 bp GPDH fragment were used.

2.5. Western blot analysis

After washing with PBS twice, the cells were dissolved in lysis solution (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 2.5 mM EDTA, 1% Triton-X100, 1% aprotinin, and 20 mg/ml leupeptin). The cells were kept on ice for 30 min and sonicated. After the sonication, the solution was centrifuged at 22,000×*g* for 20 min. The supernatant was kept at −80 °C until use and the protein content was measured using a BCA assay kit (Pierce, Rockford, IL). Western blot analysis was carried out as previously described [7].

2.6. Immunostaining

Antibodies used for immunostaining are listed in Table 2. SHs in a 35-mm dish were used for immunocytochemistry. After washing with PBS, the cells were fixed in 70% cold ethanol. After blocking with BlockAce (Dainippon Pharmaceuticals Co., Osaka, Japan) for 30 min at RT, cells were incubated with the primary antibody for 60 min at RT. Dishes were rinsed with PBS and subsequently incubated with an Alexa^{488}-conjugated antibody (Molecular Probe, Eugene, OR) for 30 min at RT. In case of double staining, the secondary antibody was applied for 60 min. After washing with PBS, the Alexa^{594}-conjugated antibody (Molecular Probe) was applied for 30 min. Finally, cells were embedded with 90% glycerol including 0.01% *p*-phenylenediamine and 4,6-diamidino-2-phenylindole (DAPI).

For immunohistochemistry, the liver was frozen at −80 °C until use. Then 7-μm-thick sections were prepared and air-dried. The staining procedure used for the sections was the same as for immunocytochemistry. A confocal laser microscope (Zeiss, Jena, Germany) was used for observation.

2.7. D-galactosamine administration

GalN (Sigma; 75 mg/100 g body weight dissolved in PBS) was intraperitoneally given to male F344 rats weighing 150–200 g [22]. The animals were killed 1–5 days after the treatment and their livers were removed. Liver slices were prepared, immediately frozen in liquid nitrogen and kept at −80 °C until use.

2.8. Cell sorting and culture

Four days after GalN treatment, hepatic cells were isolated as described above. The isolated cells were centrifuged at 50×*g* for 1 min. The supernatant was collected and then centrifuged again. After the same procedure was repeated, the supernatant was centrifuged at 150×*g* for 5 min and the pellet was suspended in PBS containing 2 mM EDTA and 0.5% BSA. An anti-CD44 antibody (625 ng/ml) was added and, following incubation for 10 min, cells were washed