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Expression of neuritin during liver maturation and regeneration

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Abstract **Cell surface molecules are not only important for cell–cell interactions but also useful for a marker to define cell types and differentiation stages. Unlike hematopoietic system in which numerous such antigens have been identified, only a few cell surface molecules have been used to define differentiation stage of hepatocytes. In order to identify such cell surface molecules, we performed DNA microarray analysis using mRNA from fetal hepatocytes in E12.5 and E17.5 mice and cDNAs encoding a membrane protein were selected. Northern blot analysis was employed to confirm the genes upregulated during maturation of fetal hepatocytes and neuritin, a GPI-anchored protein, was found as a membrane protein expressed in hepatocytes, but not in nonparenchymal cells. Its expression increased along with liver development and the maximum expression was achieved from the neonatal to adult stage. The neuritin protein was localized in sinusoidal lumen of hepatocytes in adult liver. Partial hepatectomy transiently downregulated the expression of neuritin. The expression of neuritin mRNA in C/EBPα deficient liver was reduced to about 50% of that of wild type mice. Thus, neuritin expression is well correlated to the maturation of hepatocytes and can be a useful tool to define the differentiation stage of hepatocytes.**

Keywords: Neuritin; Liver development; Liver regeneration; C/EBPα deficient mouse

1. Introduction

The liver development can be divided into several distinct stages depending on its morphological and functional properties. Fetal liver is a major hematopoietic tissue in embryo and supports massive production of blood cells. However, fetal liver lacks most of the metabolic functions and liver acquires such functions in the perinatal stage. After birth, liver exhibits numerous metabolic functions and gradually reduces their proliferation potential. Interestingly, while fully matured liver is a quiescent organ, it has an extraordinary capacity to regenerate upon liver damage. Thus, there are dramatic changes in the characteristics of the liver during the transition from fetal to adult stages and also during liver injury and regeneration. Hepatocytes in each stage express a set of specific proteins and the earliest hepatocytes, i.e., hepatoblasts, which are precursors of hepatocytes and biliary epithelial cells, are known to express α-feto protein (AFP) and albumin [1,2]. Suzuki et al. [3] described prospective isolation of liver progenitors using a combination of mAbs against c-Met, c-Kit, CD49f, CD29, CD45 and Ter 119. We demonstrated that delta-like (Dlk), a transmembrane protein with EGF repeats, also known as Pref-1, is highly expressed in hepatoblasts. Its expression is downregulated along with differentiation and completely disappears after birth [4].

After the perinatal stage, hepatocytes express a number of liver specific genes, such as carbamoylphosphate synthetase-1 (CPS-1), tyrosine amino transferase, glucose 6-phosphatase, haptoglobin and so on [5,6]. Several genes including tryptophan oxygenase and serine dehydratase are expressed by terminally differentiated hepatocytes and they are downregulated in regenerating liver [7,8]. However, almost all of these inducible marker genes in liver maturation are enzymes involved in intracellular metabolic reactions or production of serum proteins. Therefore, identification of cell surface proteins, which are upregulated during liver maturation may provide not only insight into the understanding of the liver maturation, but also useful markers for analysis and isolation of specific cell populations with cell sorters.

In this study, we show that neuritin, which was previously reported as a GPI-anchored membrane protein expressed in neural cells, is expressed in mature hepatocytes in a C/EBPα dependent manner.

2. Materials and methods

2.1. Mice

All experiments were performed using wildtype C57BL/6CrSlc mice (Nihon SLC, Hamamatsu, Japan) and C/EBPα deficient mice [9]. For tissue blots, brain, heart, lung, liver, kidney, spleen, skeletal muscle (s. muscle) and placenta were taken from adult mice at the age of 8–12 weeks. Livers were also obtained from E12.5, E14.5, E17.5,

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neonatal (days 0–1) and adult (10–12 weeks) mice for Northern blot analysis. For partial hepatectomy, mice were subjected to conventional 70% partial hepatectomy under anesthesia [10]. C/EBPα deficient fetal livers were obtained from E16.5–E18.5 fetuses.

2.2. Microarray analysis

Fetal hepatocytes from 200 livers of E12.5 mice and 50 livers of E17.5 mice were isolated by anti-Dlk antibody and autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) [4]. Total RNA extracted by using TRIZOL (Invitrogen, Carlsbad, CA) was used for microarray analysis with GEM2 Microarray (Incyte Genomics, Wilmington, DE) carrying 9514 clones.

2.3. Northern blot analysis

Total RNA was prepared from various organs using TRIZOL (Invitrogen). Ten micrograms of total RNA from each samples were separated by electrophoresis on a 1.5% agarose gel containing 2% formaldehyde. RNA was transferred to a positively-charged Nylon Membrane (Roche Diagnostics, Basel, Switzerland) and was hybridized with digoxigenin (DIG)-labeled cDNA probes for neuritin, CPS-1, tissue inhibitor of matrix metalloproteinase-2 (TIMP-2), cyclin D1 and GAPDH. Membrane was further incubated with alkaline phosphatase-labeled anti-DIG antibody (Roche Diagnostics). Blots were developed with CDP-star (New England Biolabs, Beverly, MA). For quantitative analysis, NIH image 1.62 (National Institute of Health, MD) was used to measure densities of developed bands. The intensity of the bands for neuritin, CPS-1 or cyclin D1 was normalized by that of GAPDH in the same sample or that of ethidium bromide staining of ribosomal RNA.

2.4. Immunohistochemistry

Frozen sections (8 μm) of adult liver were incubated with anti-neuritin polyclonal antibody (AF283, R&D systems, Minneapolis, MN) followed by biotin-conjugated anti-rabbit IgG. Anti-intercellular adhesion molecule-1 (ICAM-1) monoclonal antibody (KAT-1, Caltag laboratories, Burlingame, CA) and biotin-conjugated anti-mouse IgG were also used for ICAM staining. Immunoreactive proteins were visualized by using Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA). Counter staining was performed with Mayer's hematoxylin solution (Wako Pure Chemicals, Osaka, Japan).

3. Results

3.1. Expression of neuritin in liver

To identify genes encoding a membrane protein, which are regulated in liver development, we performed DNA microarray analysis of fetal hepatocytes in E12.5 and E17.5 livers. Fetal hepatocytes from each embryonic day were positively selected by using anti-Dlk mAb and mRNA from each population was used for microarray analysis. Comparison of the gene expression profiles of the two cell populations led us to find 218 genes that were differentially expressed. Among those genes, we focused our attention on neuritin because it is a GPI-anchored membrane protein.

To confirm the expression of this protein in liver, we extracted total RNA from adult mouse tissues and examined neuritin expression by Northern blot analysis (Fig. 1). Neuritin was highly expressed in brain and liver and weak expression was observed in lung and heart.

3.2. Neuritin expression increases along with liver maturation

To address whether the expression level of neuritin is altered in liver maturation, we performed Northern blot analysis using total RNA extracted from livers at several stages, E12.5, E14.5, E17.5, neonatal and adult. Neuritin was hardly detected in E12.5 liver and weakly expressed in E14.5 liver. The expression level increased significantly along with liver development

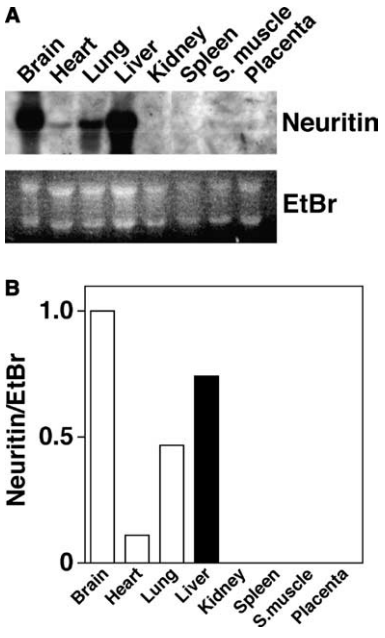

Fig. 1. Liver is a neuritin expressing tissue. (A) Neuritin expression in adult mouse tissues was confirmed by Northern blot analysis. Total RNA of adult tissues was extracted from brain, heart, lung, liver, kidney, spleen, skeletal muscle and placenta. Ten micrograms of total RNA was blotted and probed with DNA probe for neuritin. Expression level of liver was comparable with that of brain. Neuritin expression in lung and heat was also detected. (B) The intensity of the neuritin bands was normalized by ethidium bromide staining of ribosomal RNA.

and became the maximum in the neonatal to adult stage (Fig. 2), indicating that neuritin is gradually upregulated in liver development.

Recently, it has been shown that neuritin expression in mouse is regulated by male hormones [11]. We therefore compared the neuritin expression levels in male and female mouse, by Northern blot analysis. The degree of neuritin expression in female liver was almost equal to male one (Fig. 2), suggesting that male hormones do not affect neuritin expression in normal adult liver.

3.3. Expression of neuritin in hepatocytes

Liver consists of hepatocytes and various nonparenchymal cells. To identify the cell type that expressed mRNA for neuritin in adult liver, hepatocytes and nonparenchymal cells were separated by two-step collagenase perfusion method [12]. Total RNA from each population was subjected to Northern blot analysis (Fig. 3). CPS-1 is a key enzyme of ornithine cycle and known to be specifically expressed in hepatocytes but not in nonparenchymal cells [13], whereas TIMP-2 is expressed in nonparenchymal cells in liver [14]. Expression of CPS-1 and TIMP-2 indicated that hepatocytes and nonparenchymal cells were well separated. Because neuritin was detected only in RNA from hepatocytes, neuritin is specifically expressed in hepatocytes.

3.4. Localization of neuritin at the sinusoidal lumen of hepatocytes

Frozen section of adult liver was stained immunohistochemically with anti-neuritin antibody to confirm the expression in adult liver (Fig. 4). Interestingly, the staining signal was