p75 Neurotrophin Receptor Is a Marker for Precursors of Stellate Cells and Portal Fibroblasts in Mouse Fetal Liver

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Background & Aims: **Hepatic stellate cells (HSCs) and portal fibroblasts (PFs) are 2 distinct mesenchymal cells in adult liver. HSCs in sinusoids accumulate lipids and express p75 neurotrophin receptor (p75NTR). HSCs and PFs play pivotal roles in liver regeneration and fibrosis. However, the roles of mesenchymal cells in fetal liver remain poorly understood. In this study, we aimed to characterize mesenchymal cells in mouse fetal liver.** ***Methods:*** **We prepared an anti-p75NTR monoclonal antibody applicable for flow cytometry and immunohistochemistry. p75NTR$^+$ cells isolated from fetal liver by flow cytometry were characterized by reverse-transcription polymerase chain reaction, immunohistochemistry, and cell cultivation. Lipid-containing cells were visualized by Oil-red O staining.** ***Results:*** **p75NTR$^+$ cells in fetal liver were clearly distinct from endothelial cells and showed characteristics of mesenchymal cells. At embryonic day (E) 10.5, p75NTR$^+$ cells were present at the periphery of the liver bud in close contact with endothelial cells, and spread over the liver at E11.5. With the formation of the liver architecture, they began to localize to 2 distinct areas, parenchymal and portal areas, and lipid-containing p75NTR$^+$ cells increased accordingly. p75NTR$^+$ cells around portal veins were adjacent to cholangiocytes and expressed Jagged1, a crucial factor for the commitment of hepatoblasts to cholangiocytes. By cultivation, p75NTR$^+$ cells showed features of adult HSCs with markedly increased expression of glial fibrillary acidic protein and α-smooth muscle actin.** ***Conclusions:*** **p75NTR$^+$ mesenchymal cells in fetal liver include progenitors for HSCs and PFs, and the anti-p75NTR monoclonal antibody is useful for their isolation.**

The liver consists of 2 types of epithelial cells, hepatocytes and cholangiocytes (biliary epithelial cells), and various nonparenchymal cells that include hematopoietic, endothelial, and mesenchymal cells. There are 3 major mesenchymal cells in the liver (ie, hepatic stellate cells [HSCs], portal fibroblasts [PFs], and vascular smooth muscle cells residing in the walls of portal veins, arteries, and central veins). HSCs, also known as *Ito cells*, *fat-storing cells*, or *lipocytes*, are pericytes found in the perisinusoidal space between sinusoids and hepatocytes in adult liver. Protrusions extending from HSCs wrap sinusoidal endothelial cells that constitute the sinusoidal capillary network in the liver. By contrast, PFs are present in the connective tissue around portal vessels and bile ducts. In normal liver, HSCs are in a quiescent state and contain vitamin A–rich lipid droplets, constituting the largest reservoir of vitamin A in the body,[1] whereas PFs do not store lipid droplets. Although HSCs express desmin but not elastin, PFs show an inverse expression pattern.[2] In injured liver, HSCs become activated and transform into myofibroblast-like cells that secrete various cytokines to contribute to liver regeneration.[3,4] Those activated HSCs are enlarged, lack lipid droplets,[5,6] and express α-smooth muscle actin (α-SMA).[7,8] Upon prolonged liver injury, the transformed myofibroblast-like cells produce a large amount of extracellular matrix, leading to fibrosis.[1,9] Similarly, the activated PFs express α-SMA and differentiate into myofibroblasts, which are involved in the early stages of biliary fibrosis.[10,11] Because HSCs and PFs play pivotal roles in liver repair and diseases, the nature of these intriguing cell types has been a subject of intense research. HSCs show features of both mesenchymal cells and neural/neuroendocrine cells by expressing vimentin, desmin,[12] and α-SMA, as well as glial fibrillary acidic protein (GFAP),[13] nestin,[14] neural cell adhesion molecule,[15] and synaptophysin,[16] respectively. Because HSCs show mesenchymal and neuronal features, it has been postulated that their origin is related to the neuronal lineage (ie, neural crest cells). However, Cassiman et al[17] reported that HSCs are not derived from the neural crest. Moreover, it was recently reported that

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; CK, cytokeratin; Dlk, Delta-like protein; E, embryonic day; FCM, flow cytometry; Flk-1$^+$, fetal liver kinase-1; GFAP, glial fibrillary acidic protein; HSC, hepatic stellate cell; IHC, immunohistochemistry; Lyve1, lymphatic vessel endothelial hyaluronan receptor 1; mAb, monoclonal antibody; PF, portal fibroblast; p75NTR, p75 neurotrophin receptor; RT-PCR, reverse-transcription polymerase chain reaction.

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bone marrow cells become HSCs in adult liver.[18] Although the distinction between adult HSCs and PFs has now been shown,[2] the nature and origin of these mesenchymal precursors remain unclear.[19]

Hepatocytes and cholangiocytes derive from hepatoblasts, which emerge from the foregut endoderm and invade into the septum transversum mesenchyme to form the liver bud.[20] It was shown that the proliferation of hepatoblasts requires endothelial cells using explant cultures from mutant mouse embryos defective in the development of endothelial cells.[21] Because hepatocytes and HSCs interact intimately in adult liver and bile ducts are formed along with the portal veins consisting of endothelial cells and mesenchymal cells, they are speculated to interact at a certain stage of development. However, it still remains unknown when mesenchymal cells develop and whether they affect the proliferation and/or differentiation of hepatoblasts. Because adult HSCs contain numerous vitamin A–rich lipid droplets, low-density cells separated from the nonparenchymal cells in normal liver by centrifugation through various density media have been used to study HSCs.[22–26] However, this method is not applicable to the preparation of fetal HSC precursors because they do not store enough lipid droplets for separation. In addition, a lack of HSC markers for cell sorting has prevented the investigation of HSCs in liver development. Therefore, little information is available on fetal HSCs and/or their precursors and their characterization requires the prospective isolation of the fetal liver cell populations. To this end, we generated many monoclonal antibodies (mAbs) against fetal liver cells and selected those that recognize cell surface proteins. One of those mAbs recognized p75 neurotrophin receptor (p75NTR), a receptor for neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor, neurotrophin 3, and neurotrophin 4/5. Although p75NTR is expressed mainly in the nervous system,[27] it is also expressed in nonneural tissues, some tumor cells, and normal mesenchymal precursor cells of myoid cells in testis.[28] In addition, it was shown previously that human and rat HSCs expressed p75NTR[29] and that activated HSCs expressing p75NTR undergo apoptosis in response to nerve growth factor in vitro.[30–32] Recently, Passino et al[33,34] reported that depletion of p75NTR in mice exacerbated liver pathology and inhibited hepatocyte proliferation in vivo. Furthermore, they showed that ligand-independent p75NTR signaling to Rho enhanced the differentiation of HSCs into regeneration-promoting cells that support hepatocyte proliferation in diseased liver. However, the expression of p75NTR in fetal liver and the nature of p75NTR$^+$ cells remained unknown. Here, we show p75NTR expression in fetal liver at an early stage of development, and characterize the p75NTR$^+$ cells isolated from mouse fetal liver. Our data suggest that the p75NTR$^+$ cell population contains mesenchymal cell precursors for HSCs and PFs. Furthermore, we show that the p75NTR$^+$ cells in the periportal area could contribute to the formation of intrahepatic bile ducts in the late gestational stage.

Materials and Methods

Mice

C57BL/6 mice (Nihon SLC, Hamamatsu, Japan) were used for all the experiments. All experiments with animals were approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

Generation of mAbs and Identification of Their Antigens

Anti-p75NTR (clone 25-8), anti–Delta-like protein (Dlk) and antilymphatic vessel endothelial hyaluronan receptor 1 (Lyve1) mAbs were generated by immunization of a rat with mouse fetal hepatic cells. Hepatic cells were dissociated by collagenase from embryonic day (E) 14.5 mouse liver,[35] and used for the immunization after the depletion of CD45$^+$/TER119$^+$ hematopoietic cells by magnetic beads (Dynabeads; Invitrogen Corp, Carlsbad, CA). Hybridomas were established by the fusion of lymphocytes and P3X myeloma cells with polyethylene glycol as described previously.[36] To identify antigens, retrovirus-mediated expression cloning was performed as described previously.[37] To confirm the specific reactivity, mouse p75NTR, Dlk, or Lyve1 complementary DNA (cDNA) were expressed in Ba/F3 cells by a retroviral vector, pMX/IRES-GFP,[38] and the transfectants were stained with each purified antibody, and analyzed with flow cytometry (FCM).

Antibodies

In addition to the mAbs we prepared, we used 2 kinds of polyclonal antibodies against p75NTR; rabbit anti-mouse p75NTR polyclonal antibody (AB1554) (CHEMICON International Inc, Temecula, CA) and goat anti-mouse nerve growth factor receptor/tumor necrosis factor receptor superfamily, member 16 polyclonal antibody (AF1557) (R&D Systems, Minneapolis, MN) for immunocytochemistry and immunohistochemistry (IHC). The characteristics of each anti-p75NTR antibody and information of the other antibodies used are described in supplementary Figure 1 and the supplementary Materials and Methods section (see supplementary material online at www.gastrojournal.org).

Preparation of Liver Cells and Flow Cytometry

Single-cell suspensions were prepared from mouse E11.5 and E14.5 liver according to the method of Kamiya et al.[35] Aliquots of cells were blocked with anti-Fc gamma receptor III antibody, co-stained with fluorescein- and biotin-conjugated antibodies, washed, incubated with allophycocyamin-conjugated streptavidin, and analyzed by FACSCalibur (Beckon Dickinson, San Jose, CA). Dead