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ORIGINAL ARTICLE

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Potential treatment of liver-related disorders with *in vitro* expanded human liver precursors

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Abstract Inherited deficiencies in critical components of metabolic pathways are the primary cause of many liver and lysosomal disorders, most of which are incurable. Stem cell transplantation may offer a new type of treatment for these diseases. We have isolated hepatocyte precursors from human fetal livers. These cells were highly proliferative *in vitro* in media with or without serum. Expanded hepatocyte precursors expressed endoderm and early hepatocyte markers. The precursors synthesized a large number of molecules related to human metabolic diseases and released some of them into the environment. In a homing test, these cells migrated preferentially into the liver. When transplanted into fetal sheep liver, they incorporated into the liver tissue and differentiated into hepatocytes. Transplantation of the liver precursors to α-L-iduronidase-deficient mice partially corrected the enzyme deficiency. Data from these studies suggest that *in vitro* expanded human liver precursor cells are a potential cell source for the treatment of liver- and lysosome-related disorders.

Key words human · liver · lysosome · hepatocyte · transplantation · stem cell

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Introduction

Inherited hepatic and lysosomal disorders disrupt pathways in biochemical, metabolic, or signal transduction processes and affect a large population worldwide. Some disorders in this category can be treated with liver transplantation. However, the number of patients who have benefited from transplantation is limited due to the shortage of healthy donors (Sukhikh and Shtil, 2002). Hepatocyte transplantation has been developed and yielded encouraging results in treating select disease types, such as glycogen storage disease type I (Muraca et al., 2002; Muraca and Burlina, 2005) and Crigler–Najjar syndrome type 1 (Fox et al., 1998). Compared with liver transplantation, hepatocyte transplantation is much less invasive and less expensive. With improvement, it may become an alternative to liver transplantation (Strom et al., 1997).

Sources of appropriate donor cells remain a central problem for a wide range of clinical applications. A large quantity of hepatocytes is often needed for each transplantation. It has been calculated that 10%–20% of the liver cell mass must be replaced to support liver failure in adults (i.e., 10–15 billion cells or 100–150 g of hepatocytes) (Haghighi et al., 2004). However, differentiated hepatocytes proliferate poorly in culture and rapidly lose hepatocyte-specific functions. Immortalized hepatocyte lines (Kobayashi et al., 2000; Li et al., 2005) have the potential to provide an inexhaustible cell source; however, the bio-safety and differentiation potential of these cell lines are yet to be fully established. Currently, hepatocytes used for transplantation are isolated from either non-transplantable (Bilir et al., 2000) or fetal (Habibullah et al., 1994) livers. While the former represents a very limited resource, the latter is often too small to provide enough cells. In those cases where pooled fetal liver cells must be used, complications to the host immune system have not been evaluated.

Embryo and fetal livers contain a large number of precursor cells with enormous proliferation potential. If these precursors can be expanded in culture and still maintain the potential for hepatic differentiation, they can be a cell source for disease treatment. We show in this study that precursor cells isolated from embryonic or fetal livers proliferate extensively in culture. They express endoderm and hepatoblast markers, as well as a battery of enzymes critical for many metabolic pathways in the liver and lysosome, many of which are released into the extracellular environment. When transplanted into fetal sheep liver, these human liver precursor (HLP) cells incorporated into the organ and expressed hepatocyte-specific markers. In mice deficient for α-L-iduronidase (IDUA), HLP cells preferentially distributed to the liver and corrected the enzymatic deficiency partially. The results from these analyses indicate that HLP cells are a potential cell source for the treatment of hereditary liver and lysosomal metabolic diseases.

Materials and methods

Human fetus and embryo

The protocol for isolating precursor cells from aborted tissues was in accordance with the guidelines issued by the Chinese Ministry of Health and approved by the Ethical Committee of Xinhua Hospital. Human embryos and fetal tissues were obtained from the Department of Obstetrics and Gynecology at Xinhua Hospital, Department of Obstetrics and Gynecology at the Central Hospital of Putou District, and The Obstetrics and Gynecology Hospital, Fudan University, School of Medicine. Donors were negative for hepatitis and HIV viruses. The age of the fetuses was estimated by standard clinical parameters. Liver cells were isolated from embryos at 28–35 days of gestation and from fetuses at 10–12 weeks of gestation.

Cell isolation and culture

Precursors were isolated from fetal livers at room temperature (RT) if not specified otherwise. The liver was dissected and placed in a sterile dish. The tissue envelope was peeled off with fine-point forceps under an anatomic microscope. The liver was easily broken into small pieces mechanically and transferred to L15-Leibovitz medium (GIBCO/Invitrogen, Carlsbad, CA) containing 0.2% bovine serum albumin (BSA, Sigma, St. Louis, MO). The tissue was allowed to sediment for 20 min and the supernatant containing mostly red blood cells was discarded. After a wash in Hank's solution (GIBCO), the tissue was collected by centrifugation at $50 \times g$ at 4°C for 5 min and seeded into a 10 cm dish that had been coated with gelatin (0.1% for at least 1 hr, GIBCO). The tissue was incubated at 37°C in 5% CO_2 for 1 hr to promote adherence, after which Dulbecco's minimal essential medium (DMEM, GIBCO/Invitrogen) supplemented with 10% fetal bovine serum (FBS, PA), 100 U/ml penicillin (GIBCO), and 100 mg/ml streptomycin (GIBCO) was carefully added to the dish. Approximately 1 week later, the cells had grown out from the tissue. Once the dish became confluent with outgrowth, the culture was digested in 0.05% trypsin-EDTA (GIBCO/Invitrogen) for 5 min at 37°C and split into new dishes in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO_2 at 2×10^4 cells/cm^2. Cells were maintained for a prolonged period under these conditions.

To isolate precursors from embryonic liver primordial, the embryo was first removed from the chorion with fine-point forceps, washed in the 0.9% NaCl (Chemical Reagent, Shanghai, China) with 500 U/ml penicillin for 5 min. The liver bud was dissected and incubated in 0.05% trypsin-EDTA for 15 min. A serum-free culture medium consisting of 79% DMEM/F-12 supplemented with 20% knock-out serum replacement, 2 mM L-glutamine, 1% MEM-nonessential amino acids solution, 0.1 mM β-mercaptoethanol, and 6 ng/ml bFGF (all from GIBCO/Invitrogen) was added to dilute the enzyme. This medium was used throughout the isolation procedure and in long-term cultures. Cells were dissociated by passing them through a pipet several times. Dissociated cells were transferred to a 35 mm dish coated with gelatin at 2×10^4 cells/cm^2. For subculture, near-confluent cultures were split after treatment with 0.05% trypsin-EDTA for 5 min at 37°C.

Cryopreservation

Cells (5×10^6) were suspended in 1 ml ice-cold medium that consisted of DMEM, BSA fraction V (2.5% w/v), FBS (20% v/v), dimethyl sulfoxide (10% v/v) (GIBCO), and polyvinylpyrrolidone (2% w/v) (GIBCO), stored in an isopropanol (Chemical Reagent) progressive freezing container at −70°C for 1 day, and transferred to liquid nitrogen.

Immunohistochemistry

For immunochemistry, the cells were seeded on gelatin-coated plastic cover slips. The staining was performed after cells were fully spread out and firmly attached. The cells were fixed with 4% PFA (GIBCO). Nonspecific binding of the mouse or rabbit primary antibodies was blocked by incubation in 3% BSA. Polyclonal antibodies against human albumin, α-fetoprotein (αFP), α1-antitrypsin (α1-AT), and Factor IX were obtained from DAKO (Carpinteria, CA). Antibodies against HGF, c-Met, α-smooth muscle actin, and GATA-4 were purchased from Boster (Wuhan, China) and Santa Cruz Biotechnology (Delaware, CA), respectively. Monoclonal antibodies against cytokeratin (CK)18 and CK19 were purchased from DAKO and two goat anti-human HNF3β and desmin antibody from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary antibodies and non-biotin amplification (NBA, Zymed, San Francisco, CA) kits were obtained from