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Clonal Expansion of Hepatic Stem/Progenitor Cells Following Flow Cytometric Cell Sorting

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Although hepatic stem cells are believed to exist and play a critical role in developing and regenerating liver, little is known about their cell surface specificity or differentiation capabilities. To make prospective studies of hepatic stem cells possible, we established an in vitro culture system for identification and characterization of hepatic stem/progenitor cells. By combining this culture system with fluorescence activated cell sorting (FACS), a population of cells that were capable of forming large colonies and providing their descendants for relative longer period was isolated from fetal mouse livers. These data suggest that hepatic stem/progenitor cells with high proliferative potential are existent in the developing mouse liver, and that they are enriched by using flow cytometry.

Key words: Hepatocyte; Stem cell; Flow cytometry; Integrin

INTRODUCTION

Until now, there has been no definitive evidence whether hepatic stem cells exist in the liver and whether they contribute to liver development and regeneration. In the early stage of liver development, bipotent hepatic progenitor cells assumed to be capable of differentiating into both hepatocytes and cholangiocytes, namely hepatoblast, are generally believed to exist (3,14). In the adult liver, oval cells, a candidate hepatic stem cell population, were observed to emerge from the periportal area after hepatic injury when hepatocyte proliferation was inhibited (3,6,9,13). In contrast, because of the high proliferative ability of mature hepatocytes following hepatic damage, hepatic stem cells are thought to be unnecessary for liver regeneration (11). Due to the complicated knowledge, it has not been formally proven that hepatic stem cells exist. To resolve this issue, in contrast to previously performed retrospective study, a prospective study is critically required for the identification and characterization of hepatic stem cells (18,19). For this reason, we established a colony-forming assay system for hepatic stem/progenitor cells, and analyzed fetal mouse liver cells following cell fractionation using flow cytometric cell sorting.

First, we were interested in the expression of several integrin subtypes in the developing mouse liver. Integrins, which interact with the extracellular matrix, play a critical role in growth and development of normal as well as malignant liver (16,17,21,22). Histological observation of developing human liver has identified that β1 integrin and α6 integrin subunits are constitutively expressed in fetal liver and gradually limit their expression to the periportal area (2). Although laminin, a ligand of the α6β1 integrin complex (5), is expressed in liver primordium in early stages of liver development, its expression progressively decreases, and it is found only in the periportal area in adult liver (1,2,12). Because it has been suggested that hepatic stem/progenitor cells exist at the canals of Hering, a periportal site in adult liver (3,8), we hypothesized that the cells expressing both α6 and β1 integrin subunits represented hepatic stem/progenitor cells in the developing mouse liver.

MATERIALS AND METHODS

Cell Preparation

Single cell suspensions of liver cells were prepared from Balb/cA ED13.5 fetal mice (CLEA, Tokyo, Japan). Liver cells were dissociated by mechanical pipetting in staining medium (PBS containing 3% FBS). Cell viability after this treatment was more than 90% as assessed by trypan blue dye exclusion.

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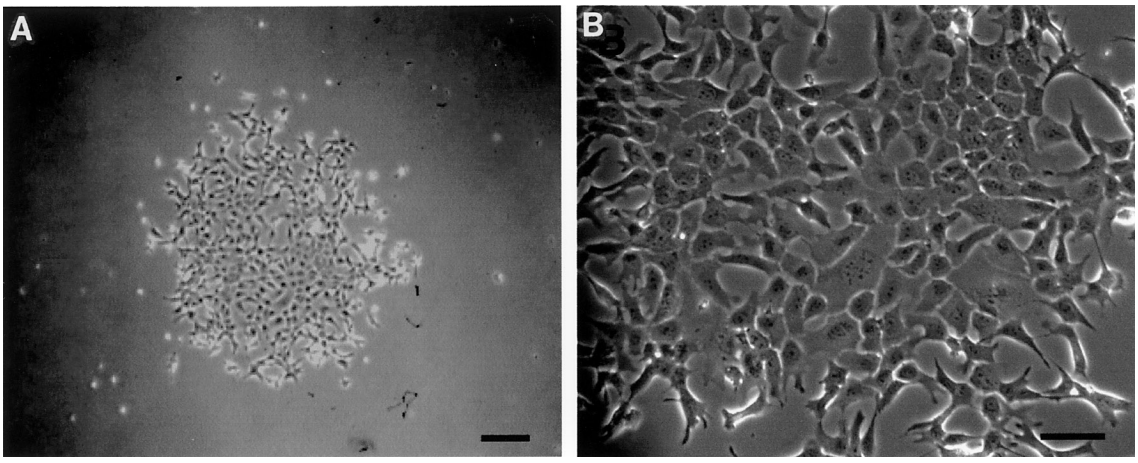

Figure 1. Formation of a large colony by a sorted cell after culture in standard medium. (A) Under clonal density culture condition (1×10^3 cells/cm^2), a single sorted H-CFU-C attached on a culture dish and formed a relatively large colony after 5 days of culture. (B) Magnified picture of (A) is shown. Scale bar: 100 μm (A), 50 μm (B).

Cell Staining and FACS Analysis

Liver cells were incubated at 4°C for 30 min with biotinylated anti-CD45 and TER119 mAb (PharMingen, San Jose, CA). After three washings with staining medium, cells were incubated with phycoerythrin (PE)-conjugated anti-CD49f mAb (PharMingen), fluorescein isothiocyanate (FITC)-conjugated anti-CD29 mAb (PharMingen), and streptavidin-labeled Texas Red (GIBCO BRL, Gaithersburg, MD) at 4°C for 30 min. Finally, cells were washed three times and resuspended in staining medium containing propidium iodide (PI) (5 μg/ml). The labeled cells were analyzed and separated with FACS-vantage (Becton Dickinson, San Jose, CA). Establishment of the gate was based on the staining profiles of the negative control.

Cell Sorting by FACS

After gating out CD45$^+$ or TER119$^+$ hematopoietic cells in the ED13.5 fetal mouse liver cells, sorting gates were set for CD49f$^+$ CD29$^+$ cell subpopulation. Sorted cells were plated on laminin-coated six-well plates (Becton Dickinson) at a density of 1×10^3 cells/cm^2. Residual erythrocytes, debris, doublets, and dead cells were excluded by forward scatter, side scatter, and PI gating. Cell viability of sorted cells was more than 85% as assessed by trypan blue exclusion.

Cell Culture

Our standard culture medium is a 1:1 mixture of DMEM and F-12 (Sigma, Chemical Co., St. Louis, MO) with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), γ-insulin (1 μg/ml) (Wako, Tokyo, Japan), dexamethasone (1×10^{-7} M) (Sigma), nicotinamide (10 mM) (Sigma), L-glutamine (2 mM) (GIBCO BRL, Gaithersburg, MD), β-mercaptoethanol (50 μM) (Sigma), HEPES (5 mM) (Wako), and penicillin/ streptomycin (GIBCO BRL). Human recombinant hepatocyte growth factor (HGF) (50 ng/ml) (Sigma) and epidermal growth factor (EGF) (20 ng/ml) (Sigma) were added 24 h after initiation of culture. During culture, the cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

RESULTS

To determine if our hypothesis was acceptable, we fractionated ED13.5 fetal mouse liver cells into CD49f$^+$ CD29$^+$ cell subpopulation ($13.7 \pm 1.20\%$, mean ± SD) by FACS, using antibodies to CD49f (α6 integrin subunit) and CD29 (β1 integrin subunit), after excluding hematopoietic cells identified by the expression of leukocyte common antigen CD45 and erythroid cell antigen TER119. The colony-forming ability of cells in this subpopulation was then examined in vitro.

We established a culture condition in which single cells could proliferate and form clonal colonies up to several hundred cells. Some of the sorted cells could form colonies of various sizes during the culture period. We observed relatively large colonies consisting of more than 100 cells (Fig. 1). We provisionally called the cell that gives rise to such a large colony a hepatic colony-forming unit in culture (H-CFU-C).

Progenitor cells are generally assumed to have the potential of generating their descendants for a relatively longer period. Therefore, to examine that H-CFU-C is a candidate of hepatic stem/progenitor cells, we cultured the sorted CD49f$^+$ CD29$^+$ CD45$^-$ TER119$^-$ cells for several months and counted the number of total cells, H-

CFU-C colony, and small size colony (<100 cells) after 3, 5, 8, 13, and 20 days of culture. After plating, CD49f$^+$ CD29$^+$ CD45$^-$ TER119$^-$ cells could proliferate from day 0 to day 13 and then reach a plateau (Fig. 2A). Although the number of small size colonies gradually decreased between day 5 and day 20 (Fig. 2C), H-CFU-C colony number was unchanged by day 20 (Fig. 2B) and this stability continued for more than 2 months (data not shown). These findings indicate: 1) most H-CFU-C colonies became larger for the culture period, in contrast to degradation of small size colonies; 2) H-CFU-Cs have the capacity for long-term cell supplication; 3) "day 5" is the best point for the determination of H-CFU-C because the number of H-CFU-C colonies was stable from 5 day of culture. These results suggest that H-CFU-C is a possible hepatic stem/progenitor cell candidate in the developing mouse liver.

DISCUSSION

Most mature hepatocytes respond to the signals of liver regeneration, proliferate extensively, and reconstitute the liver mass within several weeks (4,11). If hepatic stem cells exist in the liver, what is their role when liver regeneration is induced? The candidates for hepatic stem cell, namely hepatoblast in fetal liver and oval cells in adult liver, are allowed to supply two lineage cell types: hepatocyte and cholangiocyte (3,14). But whether or not these cells have the potential to self-renew, which is the most important characteristic of stem cells, is largely unclear. If hepatic stem cells are present and continue to supply their descendants throughout life, they need to give rise to their copies by self-renewing cell divisions. To approach this issue, prospective analysis and clonal experiments are critically required. These refined methodologies could provide better understanding of hepatic stem cell features, similar to hematopoietic stem cells (10,15,20) or neural crest stem cells (7).

In this report, we prospectively identified CD49f$^+$ CD29$^+$ CD45$^-$ TER119$^-$ cells by FACS, and their proliferation potential is higher than cells in other cell subpopulations (data not shown). H-CFU-C was capable of forming a large colony during the culture period and continuously provided their descendants for more than 2 months. It suggests that H-CFU-C in fetal mouse liver is a possible candidate for hepatic stem/progenitor cells. Flow cytometric cell separation and colony-forming analysis would make it possible to purify hepatic stem cells and analyze their features, such as multilineage differentiation potential and self-renewal capability.

Our prospective study of hepatic stem cells is useful as a screening system to search for essential factors that are required for proliferation or differentiation of hepatic stem cells. These essential factors may promote liver re-

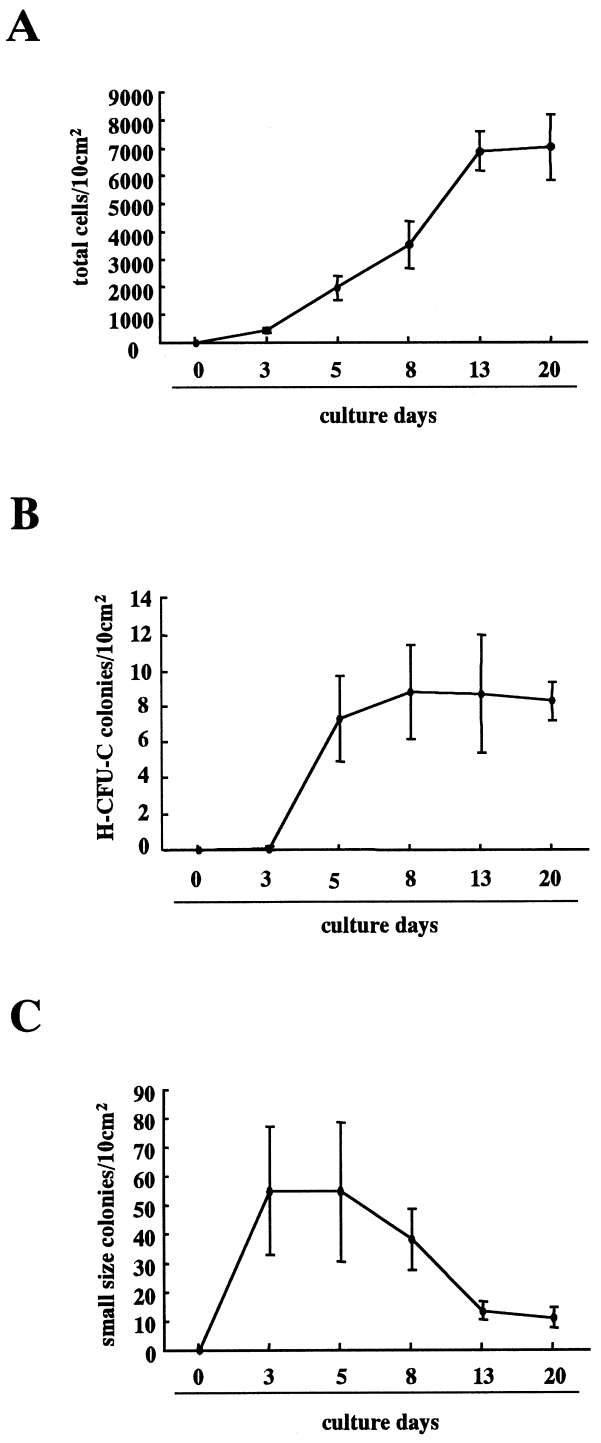

Figure 2. Time course analysis: proliferation and colony formation of the FACS-sorted CD49f$^+$ CD29$^+$ CD45$^-$ TER119$^-$ cells. Under clonal density culture condition (1×10^3 cells/cm^2), the numbers of total cells (A), H-CFU-C colonies (B), and small size colonies (C) per 10 cm^2 were counted after 3, 5, 8, 13, and 20 days of culture, respectively. Each graph represents the average of 12 dishes out of three independent experiments.

generation by in vivo manipulation of hepatic stem cells. Alternatively, for the establishment of effective gene therapy, cell therapy, and the treatment of organ failure, organ-specific stem cell identification is critically important for long-term organ reconstitution. H-CFU-C described here may be a powerful tool for regeneration therapy toward several liver disorders and could provide an opportunity to understand the system of liver development and regeneration.

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