

In Vitro Production of Functionally Mature Hepatocytes From Prospectively Isolated Hepatic Stem Cells

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Hepatocyte transplantation and artificial organ hepatic support require a number of functionally mature hepatocytes. However, their growth activity and functional behaviors are much smaller in culture after isolation from the liver. We examined whether continuously differentiating hepatocytes from multipotent hepatic stem cells that were isolated by using flow cytometry and propagated clonally in culture could be a source of clinical application. They actually gave rise to cells that were functionally equal to mature hepatocytes found in the adult liver, which secreted albumin into culture medium and metabolized harmful ammonium into urea. These data suggest that stem cell-derived hepatocytes are a useful cell source for developing therapeutic strategies, such as cell transplantation, gene therapy, and artificial liver organ to treat various liver disorders.

Key words: Hepatocyte; Stem cell; Transplantation; Artificial liver organ

INTRODUCTION

Multipotent stem cells that reside in each tissue and organ have been identified recently, and intensive studies have been conducted to identify their characteristics. In a clinical aspect, stem cells are considered as a suitable source of regenerative therapies to provide functionally mature cells within damaged tissues (6). Transplantation of isolated stem cells is expected to reconstitute an injured area by producing functionally active progenies, for example, hematopoietic stem cells can reconstitute bone marrow by giving rise to mature lymphoid, myeloid, and erythroid lineage cells after lethal irradiation (4,5). Although efficient reconstitution requires many donor cells, the number of primary isolated stem cells is too few to use them for clinical applications. To resolve this issue, it is necessary to activate proliferation of stem cells prior to transplantation in vitro.

Similar to bone marrow, neural, epidermal, myogenous, and osteogenous tissues, regenerative therapy using stem cells is expected to be useful for the treatment of hepatic failure. A previous report suggested that hepatocytes immortalized by expressing the simian virus 40T

(SV40T) antigen could provide metabolic support in acute liver failure (2). These gene-transferred hepatocytes may be a help to support temporary metabolic performance of patients awaiting liver transplantation. However, because the risk of transformation into cancer cells remains, it is required to monitor the transplanted cells for the entire life span of recipients. Expectedly, a number of transplantable mature hepatocytes should be continuously produced from hepatic stem cells that are directly isolated from the liver and propagated in culture with self-renewing cell divisions.

In our previous study, we fractionated embryonic day (ED) 13.5 fetal mouse liver cells by combining fluorescence-activated cell sorting (FACS) and monoclonal antibodies to isolate multipotent hepatic stem cells. Purified cells that express the hepatocyte growth factor receptor c-Met and weakly CD49f ($\alpha 6$ integrin subunit), but do not express c-Kit (stem cell factor receptor), CD45 (leukocyte common antigen), and TER119 (a molecule expressed on immature erythroid cells), had various stem cell activities (7). Such sorted stem cells could be clonally propagated in culture for more than 6 months, where they continuously produced hepatocytes and cholangio-

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cytes as descendants while maintaining primitive stem cell population. Although it is possible to consider these hepatic stem cells as a source of regenerative therapies toward liver failure, it is necessary to determine whether stem cells could produce functionally mature hepatocytes in vitro consecutively.

In this study, we examined functional capabilities of differentiating hepatocytes derived from hepatic stem cells in vitro. Actually, stem cells could give rise to hepatocytes capable of secreting albumin and metabolizing ammonium. Furthermore, as shown in our previous report, these stem cell-derived hepatocytes also contained binuclei in their cytoplasm and expressed functional genes found in mature hepatocytes in adult liver (7).

Taken together, these results demonstrate that purified fetal liver-derived hepatic stem cells can produce functionally mature hepatocytes continuously with their own self-renewing cell divisions in vitro. These cells thus should be considered as a useful cell source for developing therapeutic strategies, such as cell transplantation, gene therapy, and artificial liver organ, to treat various liver disorders.

MATERIALS AND METHODS

Hepatic stem cell clones were prospectively isolated from C57BL/6 ED13.5 fetal mice (CLEA, Tokyo, Japan) and were clonally cultured as described previously (7). After the initiation of culture, we maintained them in culture by replating them every 7 days. Three clones were randomly selected and those cells were used for examination. We used human recombinant HGF (Sigma Chemical Co., St. Louis, MO) and cultured cells on non-coated dishes and on laminin-, type IV collagen-, type I collagen-, fibronectin-, and Matrigel-coated dishes (Becton Dickinson, San Jose, CA) to test their effects on hepatic stem cells. Functional performances of the cultured cells, such as albumin secretion and ammonium metabolism, were examined as described previously (3,9).

RESULTS

Growth and Differentiation Activity of Hepatic Stem Cells

As a source of experiments, we sorted cells from the c-Met⁺ CD49f^{int/low} c-Kit⁻ CD45⁻ TER119⁻ cell fraction in ED13.5 fetal mouse livers by using FACS, and propagated several clones in clonal cultures as described previously (7). Our previous studies with highly enriched populations with stem cell activity showed that HGF was a critical requirement for initial colony formation (7,8). In this study, to determine a suitable condition of stem cell proliferation, we cultured cells with various concentrations of HGF on dishes coated by components of the extracellular matrix (ECM) for 5 days. Cell count-

ing after the culture of cells in such conditions showed that their cell divisions depended on the presence of HGF and on tight adhesion onto laminin, type IV collagen, and type I collagen (Fig. 1). The effect of HGF increased in a dose-dependent manner, but it reached a plateau at the concentration of 40 ng/ml.

Albumin Secretion by Neogenetic Hepatocytes Derived From Hepatic Stem Cells

We next examined albumin secretion rate of differentiating stem cell-derived hepatocytes by using a quantitative ELISA method. Albumin secretion increased significantly as stem cells differentiated, suggesting that stem cells that we isolated could continuously produce mature hepatocytes with their own self-renewing cell divisions in long-term cultures (Fig. 2A, B). In this culture, HGF promoted albumin secretion in both total cells and each individual cell (Fig. 2A, B). In addition, laminin, type IV collagen, and type I collagen also enhanced its secretion by day 20 in both total and each cell amount (Fig. 2C, D). By day 10, Matrigel could also induce its secretion in individual cells (Fig. 2D). This material, however, accelerated stem cell differentiation by significantly inhibiting their proliferation. Thus, Matrigel is not considered as an appropriate supplement for constructive production of a number of hepatocytes.

While the potential for growth induction in several components of the ECM was less than HGF (Fig. 1), they could induce differentiation of albumin-secreting hepatocytes similar to HGF. Our present results suggest that HGF promotes both proliferation and differentiation of hepatic stem cells, but laminin, type IV collagen, and type I collagen work as supportive elements for their proliferation and enhance their differentiation into hepatocyte lineage cells.

Urea Production by Functionally Mature Hepatocytes Differentiating in Hepatic Stem Cell Cultures

Mature hepatocytes can metabolize harmful ammonium into removable urea in the liver. To test the potential for ammonium metabolism in differentiating stem cell-derived hepatocytes, we estimated urea production in stem cell cultures sequentially. After the stimulation by ammonium chloride, urea product accumulated gradually in the culture medium (Fig. 3). This result clearly showed that hepatic stem cells could continuously produce functional hepatocytes that were capable of metabolizing ammonium in culture. The HGF, to a lesser extent, could also activate urea secretion in differentiating hepatocytes.

DISCUSSION

Liver regenerative therapies, particularly hepatocyte transplantation and artificial organ hepatic support, require

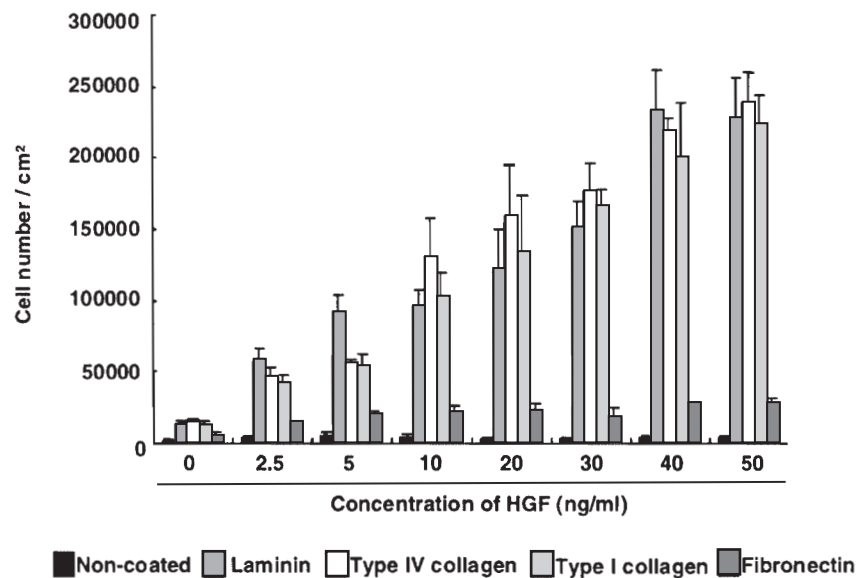


Figure 1. The HGF and components of the ECM enhanced growth activity of hepatic stem cells in culture. Isolated and propagated stem cells were plated (2×10^3 cells/cm²) on six-well plates coated by several components of the ECM and cultured with HGF (2.5, 5, 10, 20, 30, 40, 50 ng/ml) for 5 days, and then the number of cells was counted. The graph shows the average of six dishes for each culture condition. Among three independent stem cell clones, similar data were obtained.

a number of functionally mature hepatocytes as a cell source to compensate for decreased liver function. Although propagation of freshly isolated hepatocytes has been attempted extensively, it has been very difficult to proliferate them to maintain their intrinsic functions in culture. Using stem cells as a source of functionally mature hepatocytes is therefore expected to produce them continuously as a descendant *in vivo* and *in vitro*. By using flow cytometric cell separation method, we have isolated and propagated hepatic stem cells derived from the developing mouse liver in clonal cultures (7). Although they could differentiate into hepatocytes and reconstitute liver tissues after transplantation into the injured liver (7,8), it has been unreliable whether functionally mature hepatocytes could be produced from stem cells *in vitro* as well as *in vivo*.

Our present studies revealed that hepatic stem cells gave rise to cells that were functionally equal to mature hepatocytes found in the adult liver. Stem cell-derived hepatocytes had binuclei in their cytoplasm, secreted albumin, metabolized ammonium, and expressed several marker genes for mature hepatocytes. Albumin secretion and urea production of freshly isolated mature hepatocytes decreased quickly in culture (1,3,9). However, because stem cells could produce hepatocytes continuously in addition to their own self-renewing cell divisions, those of stem cell-derived neogenetic hepatocytes in-

creased gradually as culture term proceeded. Therefore, stem cell-derived hepatocytes could be used as an available cell source for transplantation or a hybrid artificial liver support whenever they are required, following thawing from frozen stocks and expansion to large volume. This therapeutic concept based on capacity of stem cells provides a theoretically unlimited source of transplantable hepatocytes without any transference of immortalizing genes into cells, and it could provide a quick and safe method to treat acute liver failures. Furthermore, because propagating stem cells could be easily infected by a retrovirus and expressed transferred genes stably *in vivo* and *in vitro* (7), they should provide a powerful tool and information for liver gene therapy by using manipulated stem cells.

Promotion of hepatocyte differentiation from stem cells *in vitro* could provide more efficient functional support with hepatic disorders by promoting hepatocyte transplantation or artificial organ hepatic support. In this study, functional behaviors in stem cell-derived hepatocytes, such as proliferation, albumin secretion, and ammonium metabolism, were enhanced by HGF, suggesting that it is an inducible factor for hepatocyte differentiation from hepatic stem cells. Several components of the ECM, such as laminin, type IV collagen, and type I collagen, also induced differentiation of stem cells to hepatocyte lineage cells. Screening of various elements that

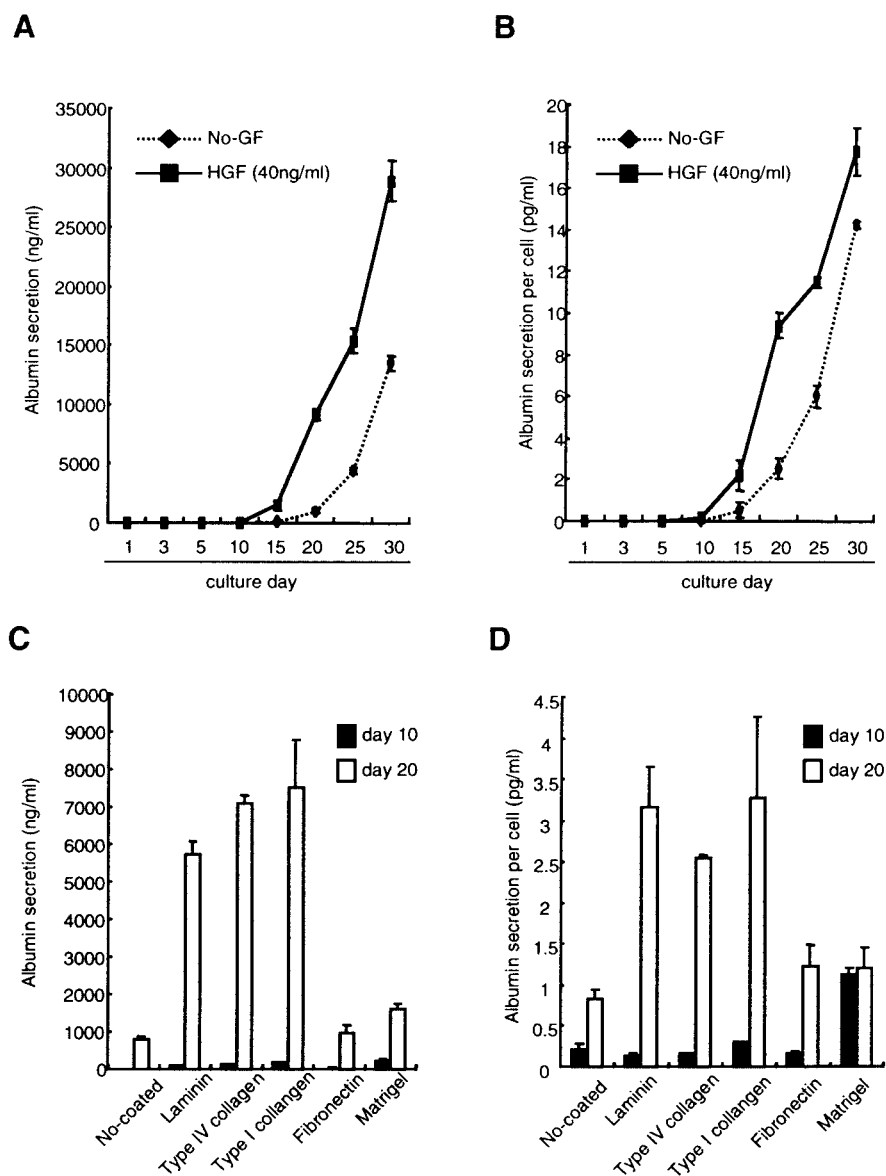


Figure 2. Albumin secretion by differentiating stem cell-derived hepatocytes. Hepatic stem cell cultures were initiated on six-well culture plates, and then quantity of albumin that was secreted into the culture medium was measured using ELISA. (A, B) In cells cultured on laminin-coated dishes with or without HGF (plating cell density: 1×10^5 cells/cm²), total amount of albumin was measured (A) and its quantity in each cell was calculated (B) at days 1, 3, 5, 10, 15, 20, 25, and 30 in culture. Note that HGF induced albumin production efficiently in differentiating hepatocytes. (C, D) When stem cells were cultured on several components of ECM (2×10^5 cells/cm²), laminin, type IV collagen, and type I collagen enhanced albumin secretion from both total cells (C) and individual cells (D) by day 20 of culture. The graph shows the average of six dishes for each culture condition. Among three independent stem cell clones, similar data were obtained.

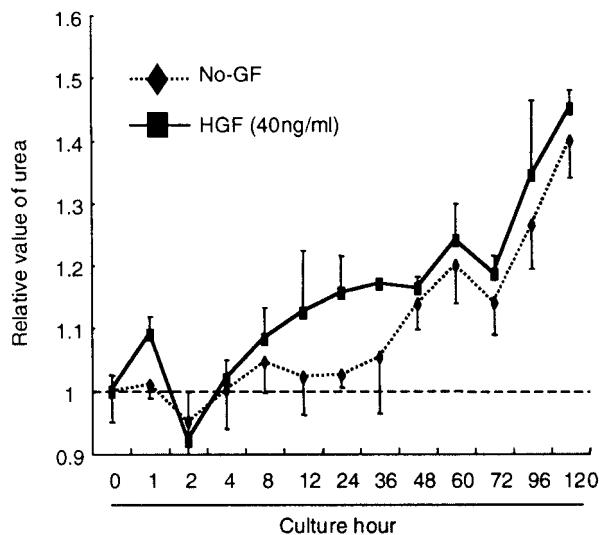


Figure 3. Urea production by stem cell-derived functionally mature hepatocytes. After the stimulation of semiconfluent cultured cells by ammonium chloride (1 mM), supernatant of culture medium was collected continuously and examined for urea secretion. The HGF also enhanced urea secretion from differentiating hepatocytes. The graph shows the average of four dishes for each culture condition. All data were normalized to the value of prestimulation. Among three independent stem cell clones, similar data were obtained.

induce hepatocyte differentiation from stem cells may discover a critical substrate that could stimulate proliferation and differentiation of endogeneous hepatic stem cells directly in the liver development and regeneration.

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