In vitro cultivation and differentiation of fetal liver stem cells from mice

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ABSTRACT

During embryonic development, pluripotent endoderm tissue in the developing foregut may adopt pancreatic fate or hepatic fate depending on the activation of key developmental regulators. Transdifferentiation occurs between hepatocytes and pancreatic cells under specific conditions. Hepatocytes and pancreatic cells have the common endodermal progenitor cells. In this study we isolated hepatic stem/progenitor cells from embryonic day (ED) 12-14 Kun-Ming mice with fluorescence-activated cell sorting (FACS). The cells were cultured under specific conditions. The cultured cells deploy dithizone staining and immunocytochemical staining at the 15th, 30th and 40th day after isolation. The results indicated the presence of insulin-producing cells. When the insulin-producing cells were transplanted into alloxan-induced diabetic mice, the nonfasting blood glucose level was reduced. These results suggested that fetal liver stem/progenitor cells could be converted into insulin-producing cells under specific culture conditions. Fetal liver stem/progenitor cells could become the potential source of insulin-producing cells for successful cell transplantation therapy strategies of diabetes.

Keywords: Hepatic stem/progenitor cell, diabetes, β-cell, dithizone staining, immunocytochemistry.

INTRODUCTION

Type I diabetes results from the progressive autoimmune response which selectively destroys the insulin-producing pancreatic β cells. Since β cells are considered to be end-stage differentiated cells, it has limited capacity to generate new β cells. Patients suffered from type I diabetes require life-long insulin therapy [1]. Islet transplantation provides new therapeutic approaches to diabetes as alternative to insulin therapy, but purified islets from 2-3 cavaderic donors are required for a successful transplantation [2, 3]. The limitation of available islets becomes the main obstacle to successful islet transplantation. Although there is evidence to show that pancreatic stem cells exist in pancreas, the pancreatic stem cells have not yet been identifiable due to lack of specific markers. Currently, pancreatic stem cells have not been purified or isolated from adult pancreas in any species.

During embryonic development, the liver and pancreatic lineages arise from the common embryonic endoderm cell population in the anterior foregut [4, 5]. The pancreas is formed by growth and differentiation of endodermal buds. Embryonic liver is a source of hepatic stem/progenitor cells. We isolated the hepatic stem/ progenitor cells from ED 12-14 mice liver with FACS and then induced to produce insulin. The nonfasting blood glucose level was reduced after transplantating the insulin-producing cells to the alloxan-induced diabetic mice.

MATERALS AND METHODS

Cell isolation

The hepatic stem/progenitor cells from ED 12-14 mice liver were isolated as previously described by Suzuki [6], with some modifications as follows: the fetal livers from ED 12-14 day Kun-Ming mice (the Medical Experimental Animal Center, Beijing) were isolated under asepsis condition and placed in phosphated-bufferd saline (PBS); the fetal liver was pressed through 200-gauge sterile stainless steel net and gently pipetted to produce single-cell suspensions. The cells were resusupended with staining medium (PBS supplement with 3% fetal bovine serum) after washing and then incubated at 4 ℃ for 30 min with phycoerythrin (PE) -conjugated anti-mouse c-Kit (rat monoclonal IgG2b), fluorescein isothiocyanate (FITC)-conjugated anti-mouse TER-119 (rat monoclonal IgG2b) and FITC-conjugated anti-mouse CD45RA (rat monoclonal IgG2b) (Santa Cruz Biotechnology). The cells were washed and resuspended in staining medium containing propidium iodide (5 μg/ml) to a final concentration of 1×10^7 cells per milliliter. Two–color flow cytometry analysis was performed on High-performance cell sorter MoFlo (Cytomation). The sorting gate was set for $CD45^-$, $TER119^-$, $c\text{-}kit^-$ cells as Fig. 1. The $CD45^-$, $TER119^-$, $c\text{-}kit^-$ cells were collected and placed in the gelatin-coated six-well plates (Costar).

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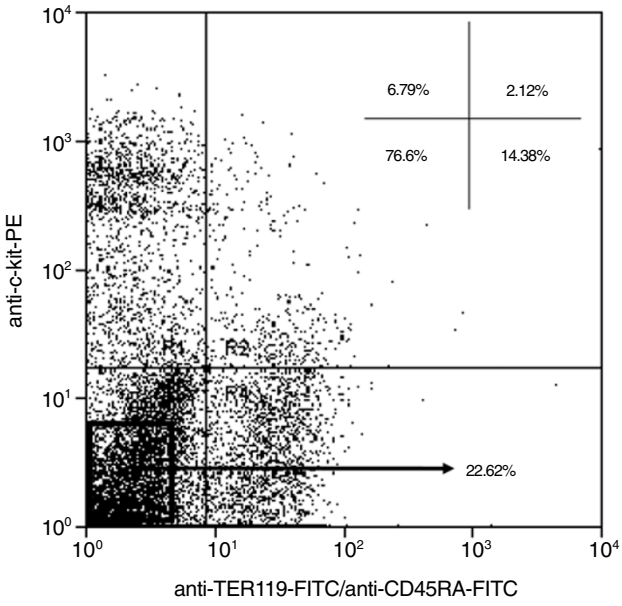

Fig. 1 Flow cytometric analysis of fetal mouse liver cells. The c-kit$^-$ CD45$^-$ TER119$^-$ cells in ED12-14 fetal mouse livers were sorted by using antibodies against c-kit, CD45, TER119. The percentage of fractionated cells is shown on the right. Representative data from six independent experiments are shown. The c-kit$^-$, CD45$^-$, TER119$^-$ cells were collected according to the flow cytometric cell sorting gate setting for the relative markers.

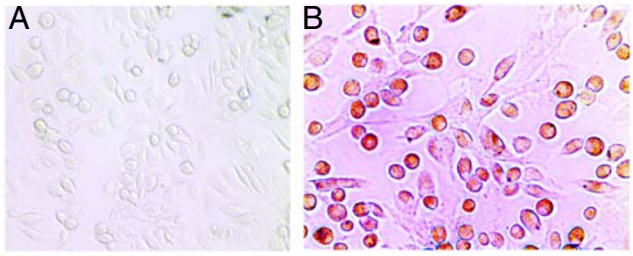

Fig. 2 Dithizone staining for insulin in cultured hepatic stem/progenitor cells (at day 15 after the initiation of culture). **(A)** Lack of staining for insulin in the negative control (without adding the dithizone). Magnification: ×200. **(B)** Presence of positive staining for insulin in the cytoplasm of induced hepatic stem/progenitor cells (in red). Data shown are represents of three independent experiments. Magnification: ×400.

Cell culture

FACS-sorted CD45$^-$, TER119$^-$, c-kit$^-$ cells were cultured in gelatin-coated six-well plates at 37 ℃ in the presence of 5% CO_2. The medium for the culture was 1:1 DMEM/F12, supplemented with 20% fetal bovine serum (FBS) (Hyclone), 1×ITS (Sigma), dexamethasone (1×10^{-7} M) (Sigma), 10 mM nicotinamide (Sigma), L-glutamine (2mM)(Gibco), β-mercaptoethanol (50 μM) (Sigma), 23 mM glucose and penicillin/streptomycin. Human recombinant hepatocyte growth factor (rHGF) (Invitrogen) (50 ng/ml) and epidermal growth factor (EGF) (Invitrogen) (20 ng/ml) were added 24 h after the initiation of culture. The medium was changed twice a week. On day 15, 30 and 40, the cells were collected by trypsinization, washed thrice with PBS and suspended in PBS at a concentration of 5×10^5 cells /ml and used for cell transplantation.

Dithizone staining

The cells were stained with dithozone (DTZ) solution (100 μg/ml of DTZ in DMEM/F12 medium without supplement) for 15 min at 37ºC at the 15th, 30th and 40th day during the whole culture period. After washing 3 times with PBS, the cells were examined with microscopy. The insulin-producing cells were stained bright red.

Immunocytochemistry

An indirect immunoperoxidase procedure was used for the immunocytochemical location of insulin in the cultured cells. Cells grew on the cover glass were rinsed three times with PBS, were fixed with 4% paraformaldehyde in PBS. Endogenous peroxidase was inactived using 3% hydrogen peroxide. Nonspecific binding was blocked with 5% normal rabbit serum. After washing with PBS, the cells were incubated with the primary antibody, rabbit anti-insulin polyclonal antibody (prepared in our laboratory), at 37ºC for 30 min. After washing with PBS, the cells were incubated with the second antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase (1:500 dilution, Vector Laboratories), at 37ºC for 30 min. After rinsing, the peroxidase substrate 3, 3'-diaminonbenzidine (DAB) was added to cover the cells, away from light at room temperature for 5 min. The staining result was examined under inverted laboratory microscope. For the negative control, the primary antibody was not used and the cells were counterstained with Wright staining. The normal mouse pancreas and liver were treated at the same time and used as positive control and negative control for the immunocytochemical analysis. They were fixed in freshly prepared 4% paraformaldehyde and processed for embedding in paraffin. Sections of paraffin embedded tissues (6 μm thick) were deparaffinized, rehydrated and stained with the primary antibody, rabbit anti-insulin polyclonal antibody, at 37ºC for 30 min. After washing with PBS, the sections were incubated with the second antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase, colorized with DAB and counterstained with Wright staining as described above.

Blood glucose in diabetes mice

Male Kun-Ming mice aged 8 weeks were purchased from the Medical Experimental Animal Center, Beijing. After fasting 15 h (free drinking of water), the mice were made diabetic with alloxan (Sigma) (180 mg/kg body weight, intraperitoneal injection). Seventy-two h after the alloxan treatment, blood samples collected from the tail were used to measure nonfasting blood glucose with Roche glucometer (Roche Diagnostics, detection range: 0.6-33.3 mM). The mice with nonfasting blood glucose between 20 mM and 28 mM were selected as recipients.

About 5×10^4 cells (some cells formed clusters), which derived from the cultured hepatatic stem/ progenitor cells, were transplanted under the left renal capsule of alloxan-induced diabetic mice. We also injected normal saline without cells into the left renal capsule of alloxan-induced diabetic mice as sham-transplantation. The nonfasting blood glucose was monitored at day 3, 7, 10, 14 and 21 posttransplant.

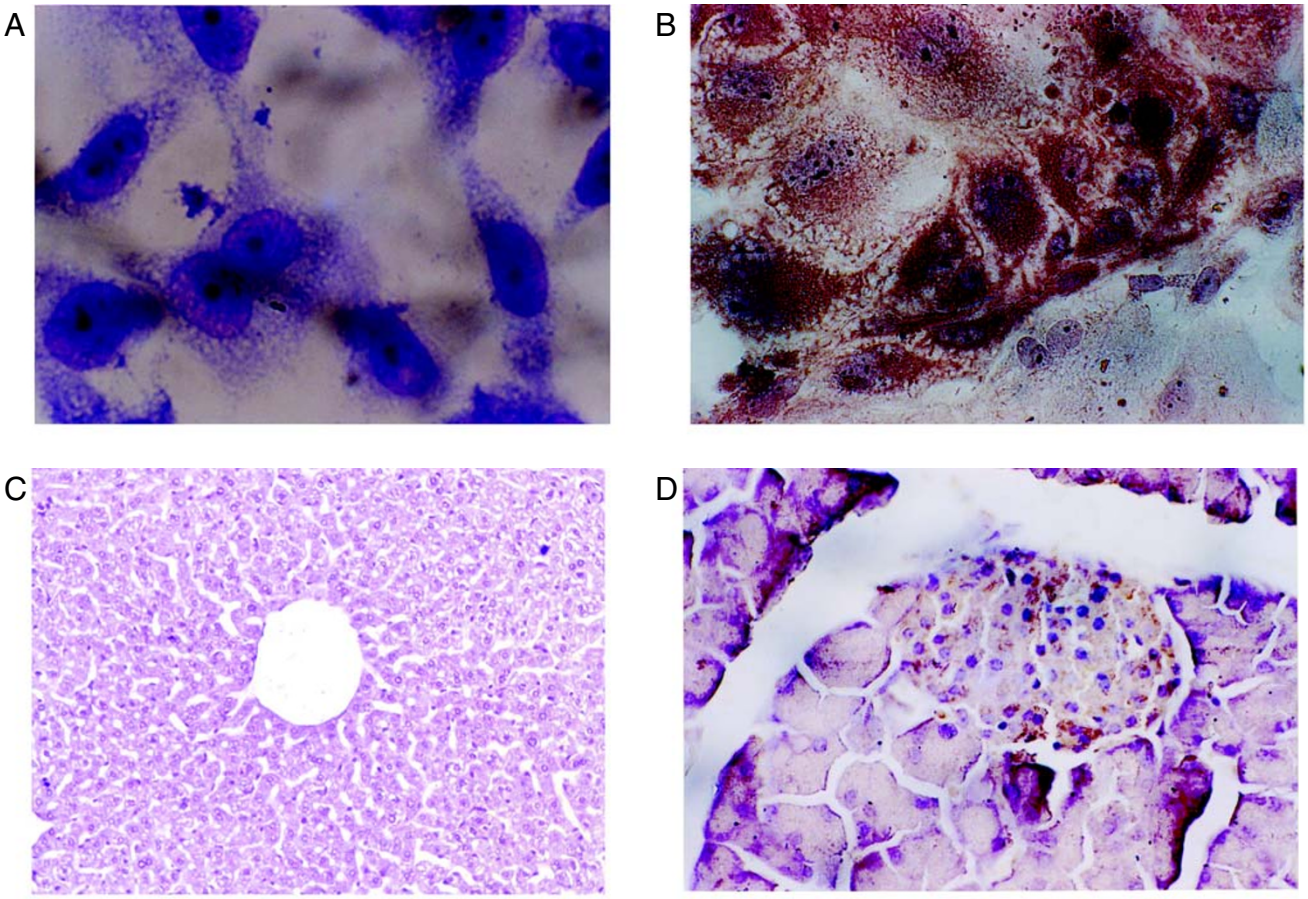

Fig. 3 Immunocytochemical staining for insulin in cultured hepatic stem/progenitor cells (at day 30 after the initiation of culture). **(A)** Lack of staining for insulin in the negative control (without adding the primary antibody). Magnification: ×600. **(B)** Presence of positive staining for insulin in the cytoplasm of induced hepatic stem/progenitor cells (in brown). Data shown are represents of three independent experiments. Magnification: ×600. **(C)** Lack of staining for insulin in normal mouse liver section. Magnification: ×200. **(D)** Positive control. Some cells in the islet stained with antibody against insulin. Magnification: ×400.

RESULTS

To exclude the hematopoietic cells from ED 12-14 fetal mouse liver, FACS-sorted CD45$^-$, TER119$^-$, c-kit$^-$ fetal liver cells were collected. The sorting gate was set as Fig. 1. The collected cell subpopulation contained hepatic stem/progenitor cells [6]. The collected cells were cultured in gelatin-coated six-well plates with the special culture medium. To assess the cultured cells developmental changes resulting from specific modifications of the culture conditions, we examined the expression of insulin by dithizone staining and immunocytochemical staining for insulin. Dithizone staining demonstrated the insulin-producing cells appeared at d 15 and exist at d 30 and 40 after the initiation of culture (Fig. 2). To confirm *in vitro* insulin production by the cultured fetal liver stem cells, we examined the insulin immunoreactivity at d 15, 30 and 40 after isolation in culture. Some of the cells were positive for insulin (Fig. 3). Our results were consistent with the previous report [6]. Suzuki *et al* reported that pancreatic, intestinal and gastric marker expression were not detectable in the sorted c-Met$^+$, CD49f$^{+/low}$, c-kit$^-$, CD45$^-$, TER119$^-$ cells. However, they became detectable by RT-PCR in the subcultured cells [6].

To demonstrate the function of the differentiated cells, the cells were transplanted under the left renal capsule of the alloxan-induced diabetic mice. The nonfasting blood glucose level was reduced till 14 d after posttransplantation (Tab. 1 and Fig. 4). At 21st day posttransplant, the nonfasting blood glucose was slightly increased, but remained lower than the level before transplantation. In contrast, the alloxan-induced diabetic mice with sham-transplantation had increasingly high levels of nofasting blood

Tab. 1 Comparison of nonfasting blood glucose levels (mM) in alloxan-induced diabetic mice after transplantation of insulin-producing cells and the sham-transplantation

Group	Before alloxan-treatment	72 h after alloxan-treatment	Time after transplantation (d)				
			3	7	10	14	21
Experimental group (n=10)	8.7± 2.1	25.2±2.8	18.7±2.3	15.7±1.8	15.0±2.5	16.0±2.0	20.8±3.1*
sham-transplantation (n=10)	9.7±2.2	25.7± 2.5	25.8±3.4	26.4±2.4	29.2±1.9§	29.0± 2.1¶	- #

Note: Results are mean ± SD. We started the experiment with 10 mice for each group. Some mice died during the experimental course, so the mouse number changed as following: * n= 8, § n=7, ¶ n=5, # n=0 . The nofasting blood glucose after transplantation of insulin-producing cells was significantly lower than that after sham-transplantation at every time point ($P < 0.05$).

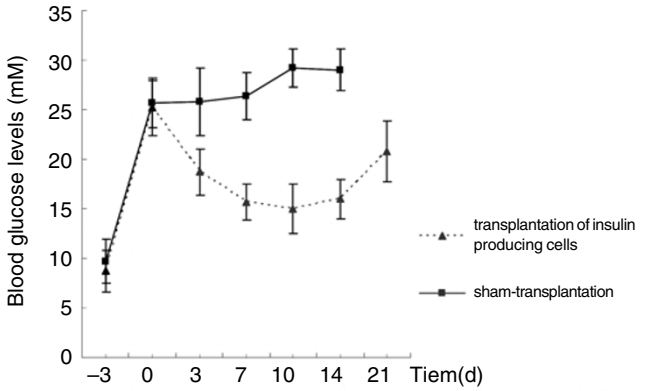

Fig. 4 Comparison of nonfasting blood glucose levels in alloxan-induced diabetic mice after transplantation of insulin-producing cells and the sham-transplantation. The figure shows the related curve between nofasting blood glucose levels and time after transplantation of insulin-producing cells (experimental group, ▲=10) or sham-transplantation (■=10).The results are mean ± SD. The nofasting blood glucose after transplantation of insulin-producing cells was significantly lower than that after sham-transplantation at every time point ($P < 0.05$). The sham-transplantation mice did not survive to 21 days after transplantaion (the mouse number at each time point refer to Tab. 1).

glucose, wasting syndrome and died in course of experiment (Fig. 4). These results indicate that FACS-sorted CD45$^-$, TER119$^-$, c-kit$^-$ fetal liver cells have the potential to convert to insulin-producing cells under specific culture condition.

DISSCUSSION

The evidence of pancreas to liver and liver to pancreas cell differentiation consist with the hypothesis that liver and pancreas arise from the common endodermal progenitor cells. Liver progenitor cells and intestinal epithelial cells could be converted into insulin-producing cells with specific culture conditions [5, 7-9]. Purified hepatic oval stem cells possess the capacity to trans-differentiate into functional endocrine cells, including insulin-producing cells [10]. When liver is injured or partially hepatectomized, the liver oval cells can be proliferated and differentiated to hepatocytes and bile duct cells. Under the normal condition, the oval cells are limited in the adult liver. But there are some disputes about the origin of oval cells.

The present study suggested that fetal liver stem/progenitor cells could be converted into insulin-producing cells under specific culture conditions. The cultured cells were deployed dithizone staining and immunocytochemical staining 15, 30 and 40 days after isolation in culture and indicated the presence of β cells. In *in vivo* study, the cells were transplanted into alloxan-induced diabetic mice. Reduction in nonfasting blood glucose level was obtained after transplantation. Nonfasting blood glucose level was slightly increased on 21st day after posttransplantation, but remained lower than the level before transplantation. The reason remains unclear, one possibility is the immunoreactivity for allograft.

To induce the hepatic stem/progenitor cells differentiate into β cells, the culture medium was supplemented with 1×ITS (Sigma), dexamethasone (1×10^{-7} M) (Sigma), 10 mM nicotinamide (Sigma), L-glutamine (2 mM) (Gibco), β-mercaptoethanol (50 μM) (Sigma), 23 mM glucose, human recombinant hepatocyte growth factor (rHGF) (Invitrogen) (50 ng/ml) and epidermal growth factor (EGF) (Invitrogen) (20 ng/ml). High glucose promotes growth and differentiation of β cells [10]. EGF promotes islet growth [11]. Nicotinamide has important effect on the differentiation of β cells from precursor. It was reported that nicotinamide could induce functional maturation of β cells [12] and protect β cells from desensitization induced by prolonged exposure to large amounts of glucose [13]. *In-vitro* and *in vivo* studies have demonstrated

that HGF is an insulinotropic factor for adult islet β cells and is able to induce insulin expression in non-β cells [14]. Nicotinamide, in combination with EGF and HGF, resulted in increase in the numbers of islets in *in vitro* culture of islet progenitor cells [13]. Suzuki and coworkers reported the hepatic stem/progenitor cells could be differentiated into hepatocytes and cholangiocytes *in vitro* culture [6] In this study we show the ability of hepatic stem/progenitor cells could be differentiated into β cells. This is consistant with the hypothesis that liver and pancreas arise from the common endodermal progenitor cells. These results provide evidence that the hepatic stem/progenitor cells may adopt different cell lineages fate depending on activation of the key developmental regulators.

The insulin-producing cells could be used as a potential cell source for successful cell replacement therapy for diabetes. Further studies are needed to purify the insulin-procuding cells and to develop the strategies for the prolongation of survival of allograft cells.

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REFERENCES

1 Mathis DL, Vence L, Benoist C. beta-Cell death during progression to diabetes. Nature 2001, **414:**792-8.
2 Bonner-Weir S, Sharma A. Pancreatic stem cells. J Pathol 2002, **197:**519-26.
3 Oberholzer J, Triponez F, Mage R, et al. Human islet transplantation: lessons from 13 autologous and 13 allogeneic transplantations. Transplantation 2000, **69:**1115-23.
4 Grompe M. Pancreatic-hepatic switches *in vivo*. Mech Dev 2003, **120:**99-106.
5 McLin VA, Zorn AM. Organogenesis: making pancreas from liver. Curr Biol 2003, **13:**R96-8.
6 Suzuki A, Zheng YW, Kaneko S, et al. Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. J Cell Biol 2002, **156:**173-84.
7 Horb ME, Shen CN, Tosh D, Slack JM. Experimental conversion of liver to pancreas. Curr Biol 2003, **13:**105-15.
8 Kojima H, Nakamura T, Fujita Y, et al. Combined expression of pancreatic duodenal homeobox 1 and islet factor 1 induces immature enterocytes to produce insulin. Diabetes 2002, **51:**1398-408.
9 Suzuki A, Nakauchi H, Taniguchi H. Glucagon-like peptide 1 (1-37) converts intestinal epithelial cells into insulin-producing cells. Proc Natl Acad Sci U S A 2003, **100:**5034-9.
10 Yang L, Li S, Hatch H, et al. *In vitro* trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci U S A 2002, **99:**8078-83.
11 Soria B. *In-vitro* differentiation of pancreatic beta-cells. Differentiation 2001, **68:**205-19.
12 Otonkoski T, Cirulli V, Beattie M, et al. A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth. Endocrinology 1996, **137:**3131-9.
13 Ramiya VK, Maraist M, Arfors KE, et al. Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. Nat Med 2000, **6:**278-82.
14 Garcia-Ocana A, Vasavada RC, Cebrian A, et al. Transgenic overexpression of hepatocyte growth factor in the beta-cell markedly improves islet function and islet transplant outcomes in mice. Diabetes 2001, **50:**2752-62.