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World J Gastroenterol 2004;10(10):1480-1486
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

Characterization and enrichment of hepatic progenitor cells in adult rat liver

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Received: 2003-08-26 **Accepted:** 2003-10-27

Abstract

AIM: To detect the markers of oval cells in adult rat liver and to enrich them for further analysis of characterization *in vitro*.

METHODS: Rat model for hepatic oval cell proliferation was established with 2-acetylaminofluorene and two third partial hepatectomy (2-AAF/PH). Paraffin embedded rat liver sections from model (11 d after hepatectomy) and control groups were stained with HE and OV6, cytokeratin19 (CK19), albumin, alpha fetoprotein (AFP), connexin43, and c-kit antibodies by immunohistochemistry. Oval cell proliferation was measured with BrdU incorporation test. C-kit positive oval cells were enriched by using magnetic activated cell sorting (MACS) .The sorted oval cells were cultured in a low density to observe colony formation and to examine their characterization *in vitro* by immunocytochemistry and RT-PCR.

RESULTS: A 2-AAF/PH model was successfully established to activate the oval cell compartment in rat liver. BrdU incorporation test of oval cell was positive. The hepatic oval cells coexpressed oval cell specific marker OV6, hepatocyte-marker albumin and cholangiocyte-marker CK19. They also expressed AFP and connexin 43. C-kit, one hematopoietic stem cell receptor, was expressed in hepatic oval cells at high levels. By using c-kit antibody in conjunction with MACS, we developed a rapid oval cell isolation protocol. The sorted cells formed colony when cultured *in vitro*. Cells in the colony expressed albumin or CK19 or coexpressed both and BrdU incorporation test was positive. RT-PCR on colony showed expression of albumin and CK19 gene.

CONCLUSION: Hepatic oval cells in the 2-AAF/PH model had the properties of hepatic stem/progenitor cells. Using MACS, we established a method to isolate oval cells. The sorted hepatic oval cells can form colony *in vitro* which expresses different combinations of phenotypic markers and genes from both hepatocytes and cholangiocyte lineage.

Qin AL, Zhou XQ, Zhang W, Yu H, Xie Q. Characterization and enrichment of hepatic progenitor cells in adult rat liver. *World J Gastroenterol* 2004; 10(10): 1480-1486
http://www.wjgnet.com/1007-9327/10/1480.asp

INTRODUCTION

It has ever been disputed whether there are stem/progenitor cells in liver, because the liver is a quiescent organ and the adult liver can regenerate by hepatocytes reentering into cell cycle after surgical resection or injury[1-3]. But it is now generally accepted that the liver contains hepatic stem cells/progenitor cells. When the ability of hepatocytes to divide and replace damaged tissues is compromised under the condition of severe and chronic liver injury caused by drugs, viruses and toxins, a subpopulation of liver cells termed oval cells, is induced to proliferate. Extensive studies in rodent models of hepatocarcinogenesis and other non-carcinogenic injury models suggest that oval cells may represent a facultative hepatic progenitor/stem cell compartment. These cells not only can be activated to proliferate but also differentiate both into mature hepatocytes and biliary epithelial cells under certain conditions[4-7]. So these hepatic stem/progenitor cells (HSCs/HPCs) are ideal sources for cell therapy such as cell transplantation or tissue engineered bioartificial organs and identification of HSCs/HPCs has become increasingly important.

Hematopoiesis and hepatic development share common stages. During fetal development, hematopoietic stem cells move out of the yolk sac and into the developing liver. Simultaneous with the appearance of hematopoiesis, hematopoietic stem cells can be detected in the fetal liver (data not shown). It is increasingly apparent that HSCs/HPCs share common characteristics with stem cells of the hematopoietic system[8,9]. C-kit is a hematopoietic stem cell receptor, and it is also expressed in hepatic oval cells[10,11]. 2-Acetylaminofluroene and partial hepatectomy (2-AAF/PH) are a traditional model to activate oval cells in rat liver[12]. We were also successful in establishment of an oval cell proliferation model treated with 2-AAF/PH. The current studies were performed to detect the markers expressed in rat oval cells and used c-kit antibody as well as magnetic activated cell sorting (MACS) to highly enrich the population of hepatic oval cells for further analysis of colony formation and characterization *in vitro*.

MATERIALS AND METHODS

Oval cell compartment proliferation/activation

Male SD rats (about 150 g) were supplied by Laboratory Animal Center of Chinese Academy of Sciences. They were fed 5, 10, 15 and 20 mg/kg body mass 2-AAF (dissolved in polyethylene glycol, Sigma) daily by oral gavage for 6 d and up to 7 d after operation and the control rats were fed saline. On the 7th d, rats were partially hepatectomized under general ether anesthesia and were not fed 2-AAF on the same day. The time points of this study were counted when partial hepatectomy was performed. One hour before the animals were killed, they received an intraperitoneal injection of BrdU (100 mg/kg body weight) (Sigma) to detect proliferation of oval cells (BrdU incorporation test). Liver tissue obtained was processed in the same manner described in immunohistochemistry methods.

Immunohistochemistry

Liver tissue was divided and fixed in 40 g/L buffered formaldehyde. All staining procedures for light microscopy

Table 1 First and second antibodies for immunohistochemistry

First antibody	Dilution	Second antibofy	Dilution	Chromogen
OV6 (mouse IgG)[1]	1:100	Goat anti-mouse IgG (HRP	1:150	DAB(brown)
		or FITC)	1:200	green fluorescence
CK19(mouse IgG)[2]	1:150	Goat anti-mouse IgG (HRP)	1:150	DAB (brown)
Albumin (rabbit IgG)	1:400	Goat anti –rabbit IgG (AP)	1:50	Fuchsine (red)
				or NBT/BCIP(blue)
AFP (mouse IgG)	1:400	Goat anti-mouse IgG (HRP)	1:200	DAB(brown)
Connexin 43(mouse IgM)[2]	1:400	Goat anti-mouse IgM (HRP)	1:200	DAB (brown)
C-kit (rabbit IgG)[3]	1:50	Goat anti-rabbit IgG (AP)	1:50	Fuchsine(red)
				or NBT/BCIP(blue)
BrdU (mouse IgG)	1:50	Goat anti-mouse IgG (HRP)	1:150	DAB(brown)

Note:1=a gift from professor Sell S, 2=from Sigma, 3=from Santa Cruz, others were from DAKO Company.

were performed on paraffin-embedded 4 μm sections. The sections were deparaffinized in xylene and rehydrated through graded alcohol. Routine histological examinations were made for liver sections stained with hematoxylin-eosin. Antigen retrieval was made by microwave - citrate buffer method, and then they were digested with trypsin (1 g/L trypsin, 1 g/L calcium chloride) at 37 ℃ for 30 min. For BrdU staining, the sections were immersed in 4N HCl for 15 min for DNA degeneration and neutralized with TBS. Single cells from suspensions were collected on glass slides by cytocentrifugation and air dried. All cells were fixed in methanol at 4 ℃ for 20 min. Endogenous peroxidase was inactivated by DAKO peroxidase blocking reagent for 10 min. The sections were incubated with 100 mL/L normal goat serum in TBS at room temperature for 20 min to block non-specific binding. For immunostaining, the primary antibody and second antibody are shown in Table 1. Double immunostaining for studies of antigen colocalization was performed with selected antibodies (OV6 *vs* albumin, CK19 *vs* albumin) using different chromogens (DAB and Fuchsine or NTB/BCIP). All antibodies were diluted with DAKO antibody diluent. Specimens were incubated with first antibody at 4 ℃ overnight, and then incubated with second antibody at room temperature for 1 h. For each antibody negative controls were performed by either blocking with appropriate nonimmune serum or by omitting the primary antibody from the protocol.

Cell sorting and culture

Rat liver cells were isolated by a two-step collagenase IV digestion method according to the protocol established by Seglen[13]. The nonparenchymal cell fraction was determined to contain the hepatic oval cell population as described by Yaswen *et al*[14]. Hepatocytes were separated from the nonarenchymal cell portion by low-speed centrifugation (50 r/min×1 min). About one third of the total liver cells were nonparenchymal cells.

Immunohistochemistry was performed on the parenchymal and nonparenchymal fractions to ensure that the cells of interest were in the nonparenchymal cell fraction. The portion of nonparenchymal cells was further purified using magnetic activated cell sorting (MACS). Cells were incubated with CD45 antiboldy (1:50) and rat erythroid cell antibody (1:200) (BD Pharmingen) at 4 ℃ for 10 min and incubated with magnetic goat anti-mouse IgG (1:5) at 4 ℃ for 15 min. MACS was performed according to the manufacturer' s recommendations (Miltenyi Biotec). The depleted fraction was incubated with c-kit antibody (1:50) and then incuabated with magnetic goat anti-rabbit IgG (1:50). The c-kit+ fraction was collected for immunohistochemistry and culture *in vitro*.

The sorted c-kit$^+$ cells were resuspended in DMEM/F-12 medium supplemented with 100 g/L fetal bovine serum, insulin-transferrin-sodium selenite, dexamethasone (1×10^{-7} mol/L), nicotinamide (10 mmol/L), L-glutamine (2 mmol/L), β-mercaptoethanol (0.1 mmol/L), HEPES (10 mmol/L) and penicillin/streptomycin. They were placed in 96-well plates, the same volume of supernatant from cultured ED13 fetal liver cells was added and incubated at 37 ℃ with 50 mL/L CO_2. Human recombinant hepatocyte growth factor (HGF, 50 ng/mL), epidermal growth factor (EGF, 20 ng/mL) and α-transforming growth factor (TGF-α, 20 ng/mL) were added 24-48 h after initiation of culture.

Phenotypic characterization of cultured cells

After 7 days, BrdU with a final concentration of 10 uM/L was added to the medium and incubated for 3 h. The cells were fixed with methanol at 4 ℃ for 20 min, and washed with PBS containing 0.5 g/L polyoxyethylene sorbitan monolaurate (Tween 20). Cytochistochemical staining was performed with Brdu, c-kit,albumin, and CK19 antibodies. The staining method was the same as immunohistochemistry.

Reverse-transcription polymerase chain reaction analysis

Characteristics of cultured cells were evaluated by reverse-transcription polymerase chain reaction (RT-PCR). mRNA was extracted from the cell colonies using a QuickPrep micro mRNA purification kit (Qiagen) according to the supplier' s recommended protocol. cDNA was synthesized using oligo-d (T)15 and Omniscript RT kit. A 20 μL of reaction mixture containing 1×Buffer RT, 0.5 mmol/L dNTP, 10 unit RNazin, 4 unit Omniscript reverse transcriptase and 1-2 μg RNA template was incubated at 37 ℃ for 1 h. PCR was done using HotStarTaq DNA polymerase in 25 μL of the reaction mixture (1×PCR buffer, 0.5 unit HotStar Taq DNA polymerase, 200 umol/L dNTP) with hepatocyte-specific primers for albumin (5' -GAG AAG TGC TGT GCT GAA GG-3' and 5' -TCA GAG TGG AAG GTG AAG GT-3'), α-fetoprotein (5' -AAC ACA TCC AGG AGA GCC AG-3' and 5' -TTC TCC AAG AGG CCA GAG AA-3'), cholangiocyte -specific primers for CK19 (5' -CTG TCT TGG TCC GGT CAC TG-3' and 5' -GGC ATC TTG GTC TGT GTC AT-3'). PCR cycles were as follows: initial denaturation at 95 ℃ for 15 min, followed by 35 cycles at 94 ℃ for 45 s, at 55 ℃ for 1 min, at 72 ℃ for 45 s, and final extension at 72 ℃ for 10 min. PCR products were separated in 20 g/L agarose gel.

RESULTS

Activation of oval cell proliferation

Oval cells could be seen in 2-AAF treated rat liver at the dosage from 10 mg/kg to 20 mg/kg weight mass 7 to 13 d after partial

heaptectomy. The peak of oval cell proliferation occured 11 d after heaptectomy. The histologic changes in liver sections from rats exposed to 2-AAF at the dosage of 10 mg/kg body mass for 6 d, followed by partial hepatectomy and sacrifice 11 d posthepatic injury are shown in Figure 1. The proliferated oval cells were small in size (approximately 10 μm) with a large nuclei to cytoplasm ratio, radiating from the periportal region forming primitive ductular structures with poorly defined lumen. The control rat liver showed complete lobular structure without oval cell proliferation (Figures 1C and D). DNA synthesis in the oval cells was examined by BrdU incorporation test, and many of the oval cells were in S-phase with their nuclei stained positive by nuclear immunohistochemical staining for BrdU. DNA synthesis in epithelial cells of the portal bile ductules was also examined by BrdU incorporation (Figure 2).

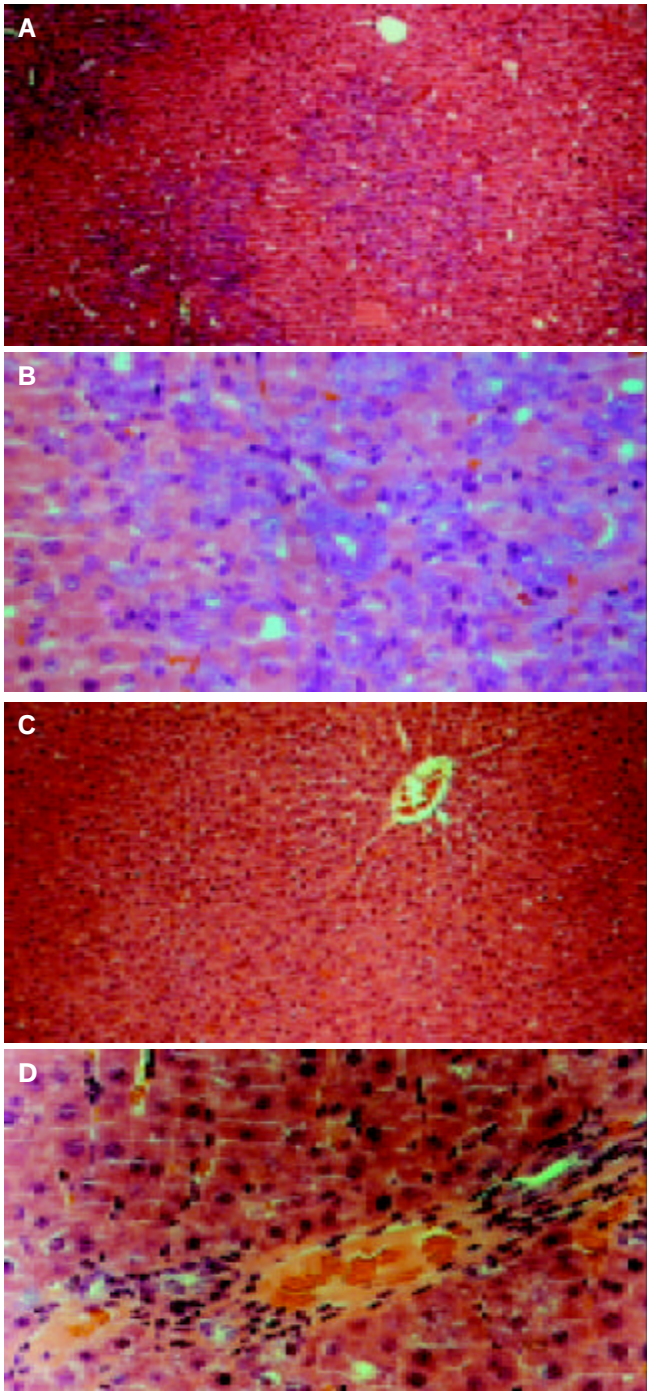


Figure 1 Rat liver sections obtained from 2-AAF/PH (11 d after operation) and control. Sections were stained with hematoxylin-eosin. (A and B) show liver sections obtained from 2-AAF/PH-treated rats at low (×100) and high (×400) magnification. Small oval cells (arrows) can be seen close proximity to proliferating bile ducts and in areas of ductular proliferation or in acinar arrangements around hepatocytes. The oval cells radiate from the periportal region, forming primitive ductular structures with poorly defined lumen. (C and D) show liver tissue from control rats at low and high magnification. Hepatoctye proliferation can be seen in the typical liver architecture. Central vein(CV) and portal triad region can be seen.

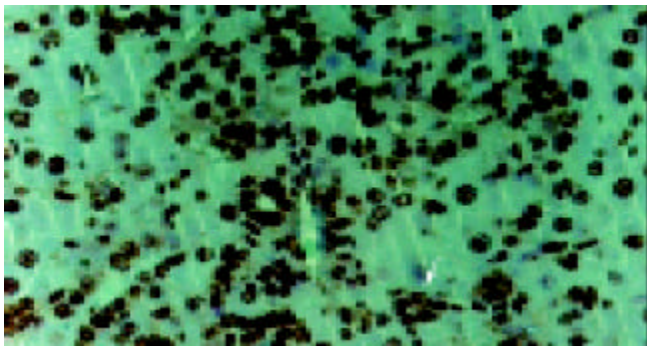
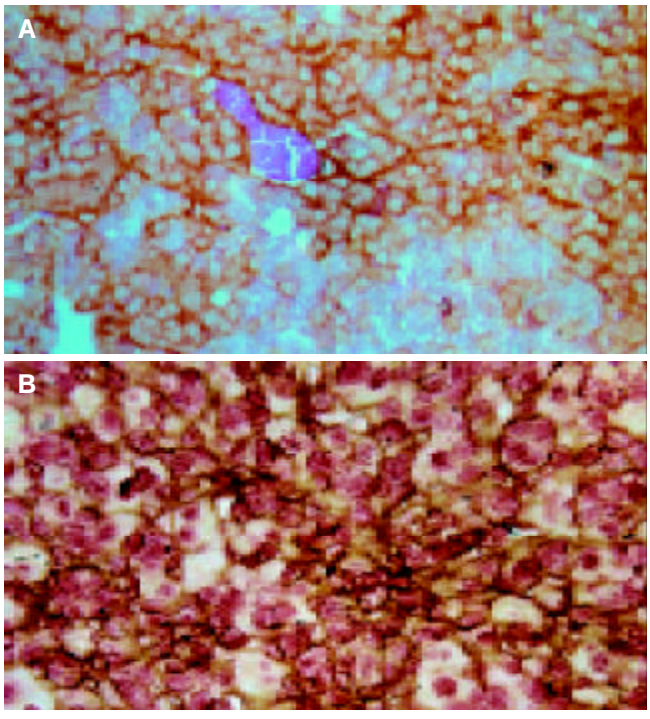


Figure 2 Immunohistochemistry of liver sections obtained from 11 2-AAF/PH rats. BrdU staining of oval cells in proliferative state. Arrows inidicate oval cells.

Immunohistochemistry for marker on activated hepatic oval cells

The sections were stained with antibodies for oval specific marker, biliary lineage marker and heaptocyte lineage markers (OV6, CK19, albumin and AFP). The oval cells generated from 2-AAF/PH treated rat liver were positive for OV6 (Figure 3 A), CK19 (Figure 3C), albumin (Figures 3B and D) and AFP (Figure 3E). Double immunohistochemistry showed that oval cells coexpressed OV6 and albumin or CK19 and albumin (Figures 3B and D). The oval cells were stained positve for connexin 43, but the sections from control rats were negative for connexin 43 (Figure 3F). In Figure 3G, oval cells expressed c-kit antigen, the ductular cells appeared to be positive with little to no staining for c-kit. No liver sections from control rat were stained positive for c-kit (Figure 3H).



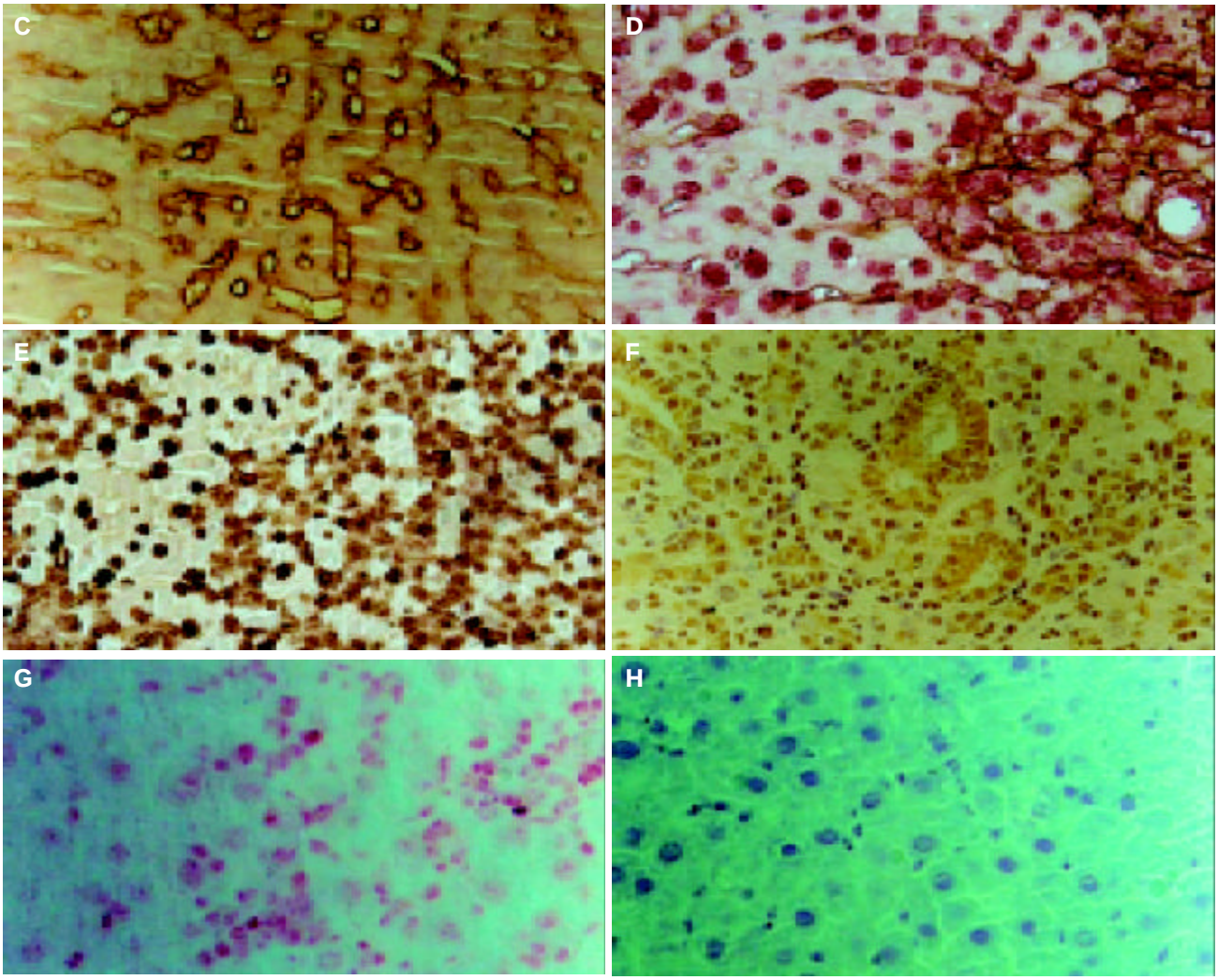


Figure 3 Immunohistochemical staining of liver sections from rat liver exposed to 2-AAF/PH (d 11) at high magnification. A: Staining for OV6, Arrows point to OV6-positive oval cells.B: Double immunohitstochemical staining for OV6 (brown) and albumin (red). C: Staining for cytokeratin19(CK19). Ductular lumen face and oval cells were positive. Arrows point ot CK19-positive cells. D: Double immunhistochemical staining for CK19 (brown) and albumin (red). Arrows point to oval cells with tow markers. E: Staining for AFP. F: Staining for connexin43, Arrows point to connexin43 positive oval cells. G: Staining for C-kit, Oval cells were stained with red. H: Negative control.

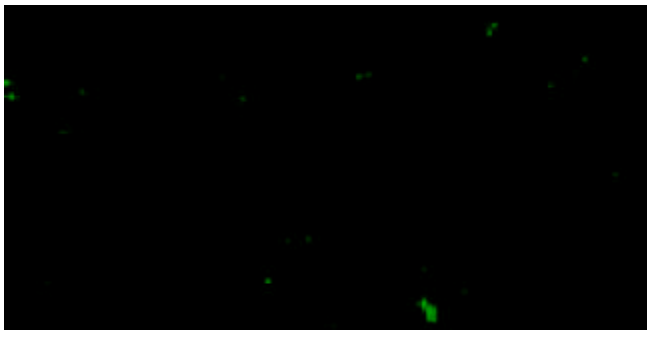
Purification and proliferation of c-kit+ oval cells

In order to define the phenotype, growth, and differentiation potential of individual c-kit$^+$ cells, we used an immunoselection and cell culture strategy. After parenchymal cells were separated from nonparenchymal cells, antibody against c-kit was used to purify c-kit$^+$ cells from nonparenchymal fraction by MACS. Immunohistochemical staining for OV6 showed that more than 90% sorted cells were positive (Figure 4).

After incubated for 24 h, most sorted c-kit$^+$ cells adhered to collagen IV coated plate. By day 3 single cells proliferated to form colonies which expanded up to several hundred cells at 2 wk (Figures 5A, B). Figure 6 shows BrdU incorporation of a cultured c-kit$^+$ oval cell colony, most cells were postive with their nuclei stained brown.

Characterization of c-kit+ oval cells

To determine the characterization of the colonies, we studied constituent cells by immunohistochemistry using albumin and CK19 as lineage markers as well as c-kit. After 1 wk, some progeny of c-kit$^+$ oval cells in the colony lost the c-kit marker of parental generation (Figure 6). Most colonies at 2 wk contained 3 types of cells, namely albumin positive cells, CK19 positive cells, both albumin and CK19 positive cells (Figure 7). RT-PCR was performed to identify the expression of genes encoding markers in both hepatocyte and cholangiocyte lineages (hepatocytes: albumin, α-fetoprotein; cholangiocytes: CK19). Almost all colonies contained mRNA of both hepatocyte-specific and cholangiocyte-specific genes at 2 wk (Figure 8). These results of RT-PCR and immunocytochemistry showed the bipotent differentiation ability of the sorted c-kit$^+$ oval cells.



Figur 4 Immunocytochemistry for OV6 on cytocentrifuged preparations of C-kit+ MACS sorted cells from 2-AAF/PH treated rats. The sorted C-kit+ cells were stained with oval cell-specific antibody OV6 (green).

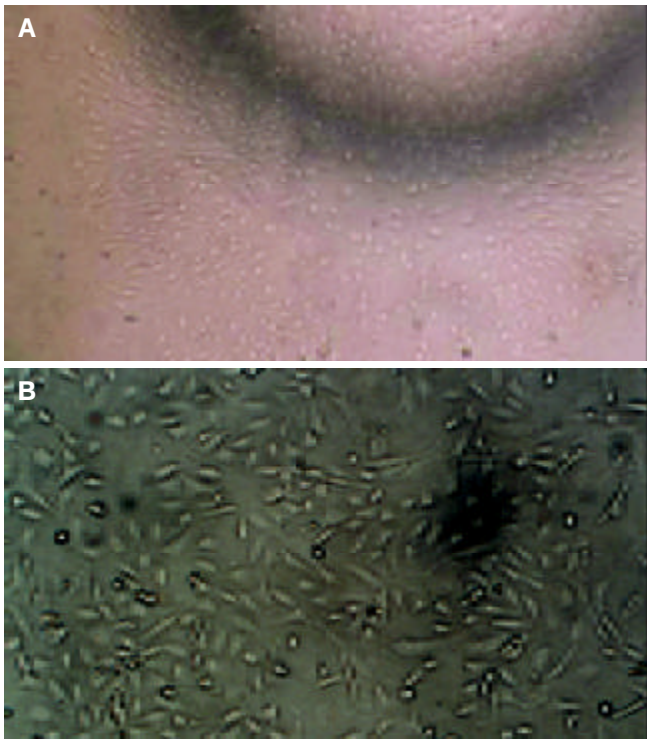


Figure 5 Phase contrast photomicrographs showing formation of a colony by a sorted C-kit+ oval cell after cultured *in vitro* for 2 wk. (A: ×100; B: ×200).

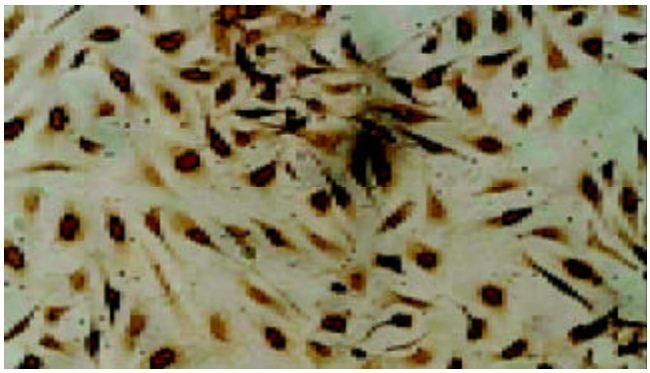


Figure 6 Double immnocytochemistry for BrdU incorporation and C-kit staining on sorted c-kit+ oval cell clony on d 7. Most cells had their nuclei stained with BrdU (arrow). Though they came from one precusor, many cells lost c-kit marker, just some of them were still c-kit positive stained blue. (arrowheads).(×400).

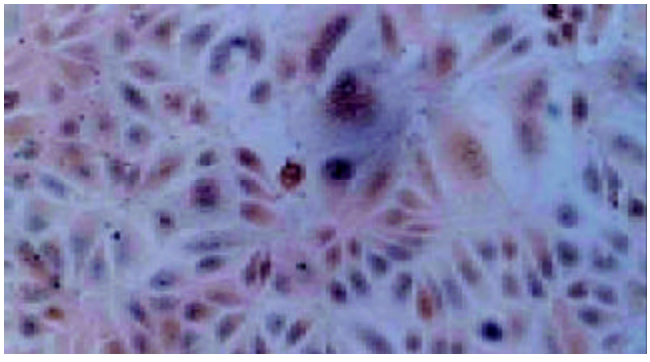


Figure 7 Dual staining of cultured sorted oval cell clony with albumin (dark blue) and CK19 (brown). Some cells were stained with both markers (Arrows) and the others were stained only one marker. (×400).

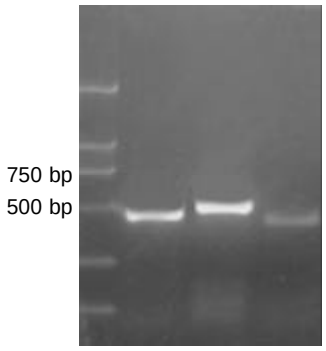


Figure 8 RT-PCR analysis of gene expression. RNA was isolated from sorted cell colony.

DISCUSSION

Since liver transplantation is the only available current therapy for end-stage liver failure and there is an ever-increasing shortage of donor livers, cell therapy from atlterative cell source might offer a new therapeutic approach against liver disease[15]. In recent years, such studies have been conducted successfully using primary hepatocytes in rodent models, and current research is being conducted to isolate progenitor or stem cells that should have the highest potential for effective liver repopulation. Therefore, identification of hepatic/progenitor cells has become increasingly important. Stem cells are defined as the cells with the self-renewing activity and capability of generating multiple types of lineage committed cells. As for the hepatocytic lineage, stem cells had to possess the ability to generate both parenchymal hepatocytes and bile duct epithelial cells[16]. After debated for many years, it has been generally accepted that the liver contains cells with stem-like properties and that these cells can be activated to proliferate and differentiate into mature hepatocytes and cholangiocytes under certain pathophysiologic circumstances[17,18]. These cells might be related to the so-called "oval cells", originally identified by Farber as immature epithelial cells with oval shaped nuclei and scant cytoplasm[19].

Activation of the oval cell proliferation could be induced by many rodent models[20-22], we chose modified Solt-Farber model to activate oval cells[12]. The model could be successfully established by 2-AAF at the dosage from 10 mg to 20 mg and the peak of oval cell proliferation was on d11 after partial heaptectomy. Immunohistochemical staining showed that the oval cells expressed oval cell-specific marker OV6, cholangiocyte lineage marker CK19 and heaptocyte lineage marker albumin and AFP. OV6 and albumin or CK19. Albumin was coexpressed in the same oval cells by double immunohistochemistry. These data suggest that the oval cells may represent a facultative hepatic progenitor/stem cell compartment. The oval cells also expressed connexin 43, but the liver cells in the control rat liver expressed connexin 32 instead of connexin 43(data not shown). Gap junctional intercellular communication (GJIC) was considered to play a key role in the maintenance of tissue independence and homeostasis in multicellular organisms by controlling the growth of GJIC-connected cells. Gap junction channels are composed of connexin molecules, connexin 43 is expressed on immature liver cells or cholangiocytes, connexin 26 and connexin 32 are expressed on hepatocytes. As hepatocytes differentiate, the proportions of connexin 43 then 26 mRNAs decrease while that of connexin 32 mRNA increases. Connexin 43 was also expressed in fetal liver cells and the expression of connexin 43 declined after birth[23]. Neveu reported that proliferating oval cells expressed diffuse connexin 43 immunoreactivity. Differentiation of oval cells into basophilic hepatocytes resulted in their alterations in Cx32 and

Cx26 expression[24].Since the oval cells were generated by the proliferation of terminal bile ducts, they formed structures representing an extension of the canals of Hering[25], and there are many common features between immature embryonic hepatic cells and oval cells such as expression of AFP and connexin 43. It is reasonable to assume that oval cells may be a direct progeny of resident undifferentiated liver stem cells. Suzuki *et al*. have identified a population of hepatic stem cells that exist in developing mouse liver , and these cells may represent the resident hepatic stem cells which possess multilineage differentiation potential and self-renewing capability[26,27]. Chen also[28] reported that transfused oval cells through caudal vein can migrate into the parenchyma of the liver and are settled there.

Hepatic progenitor cells share common characteristics with stem cells of the hematopoietic system. Classical hematopoietic markers, including Thy-1, CD34, c-kit were expressed on the surface of oval cells[8-11]. After bone marrow transplantation, a proportion of the regenerated hepatic cells was shown to be donor-derived[29]. Intravenous injection of adult bone marrow cells (c-kit^{high}Thy^{low}Lin^{-}Sca-1^{+}) in the FAH–/– mouse, an animal model of tyrosinemia type I, rescued the mouse and restored the biochemical function of its liver[30]. Crosby *et al*.[11] reported that there were c-kit^{+} cells in the periportal tract surrounding bile ducts, which coexpressed CK19. After cultured in biliary cell growth media, the isolated c-kit postive cells could form colony with the properties of cholangiocytes[11]. We also found that oval cells in the periportal area in 2-AAF/PH expressed c-kit, but hepatocytes and liver in control animal were stained negative. The evidence of a cell lineage relationship between the hematopoietic system and the liver supports the extrtahepatic origin of hepatic progenitors.

Suzuki *et al*. recently reported that CD49f^{low}c- Met^{+} c-Kit^{-} CD45^{-}TER119^{-} cell subpopulation in ED13.5 mouse fetal liver could form large colonies designated as ' hepatic colony-forming-unit in culture' (H-CFU-C), and expression of *c-kit*, *CD34* and *thy-1* became detectable in some of these colonies on d 21[26]. These data may suggest that not only exogenous hepatic stem/progenitor cells derived from circulating bone marrow express hematopoietic stem cell markers, but endogenous tissue-determined stem/progenitor cells express such markers.

C-kit stem cell receptor tyrosine kinase (KIT) and its ligand, stem cell factor (SCF) system play a crucial role in the development of oval cells. If the c-kit kinase activity was severely impaired, the number of oval cells on d 7, 9, and 13 after PH was significantly reduced to 15%, 18%, and 27% of those in control normal rats in the AAF/PH model, respectively[10]. So we chose c-kit as a surface marker to purify oval cells. After depletion of CD45+ cells and rat erythroid cells from nonparenchymal fractions, oval cells were further enriched by MACS using c-kit antibody. The result showed that more than 90% sorted cells were OV6 positive. After cultured in our special medium the sorted oval cells could form large colonies. BrdU incoporation test was positve in most progenies of oval cells on d 7, indicating that the sorted oval cells have high growth potential. At wk 2, some of the progenies lost the c-kit marker of the sorted cells. Immunohistochemical staining showed that there were 3 types of cells in the colony after cultured for 2 wk *in vitro*, that marked albumine, only CK19, and both albumin and CK19. RT-PCR on the colony showed that expression of albumin, AFP and CK19 genes was detectable. All these data suggest that the sorted oval cells are facultative hepatic stem/progenitor cells with self-renewing capacity and can differentiate along hepatocyte and cholangiocyte lineages *in vitro*, and the progeny lost stem cell marker during differentiation. Crosby *et al*. purified c-kit positive cells from cirrhotic tissue by MACS and the sorted cells could form colonies with 2 types of cells in morphology cultured *in vitro* on d 7. But the sorted c-kit^{+} or CD34^{+} cells by Crosby *et al*. could only differentiate along cholangiocyte lineage[11]. The difference might be due to the fact that they used just biliary cell growth medium.

In conclusion, we successfully isolated and purified viable c-kit positive oval cells which had high growth potential and bilineage differentiation activity from the 2-AAF/PH rat lvier. The properties of proliferation and differentiation *in vivo* are still to be studied by cell transplantation.

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Edited by Zhang JZ and Wang XL **Proofread by** Xu FM