Characterization and enrichment of hepatic progenitor cells in adult rat liver

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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.

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,[2]. 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[3-7]. 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. Simultaneously 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[8-10]. C-kit is a hematopoietic stem cell receptor, and it is also expressed in hepatic oval cells[10,11]. 2-Acetylaminofluorene 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

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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 DAKO antibody diluent. Specimens were incubated with first antibody at room temperature for 1 h. For each antibody negative controls were performed by either blocking with blocking reagent for 10 min. 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 °C 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 °C 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.

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 °C for 20 min, and washed with PBS containing 0.5 g/L polyoxyethylene sorbitan monolaurate (Tween 20). Cytochemistochemical 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 1xBuffer RT, 0.5 mmol/L dNTP, 10 unit RNazin, 4 unit Omniscript reverse transcriptase and 1-2 µg RNA template was incubated at 37 °C for 1 h. PCR was done using HotStarTaq DNA polymerase in 25 µL of the reaction mixture (1xPCR buffer, 0.5 unit HotStar Taq DNA polymerase, 200 umol/L dNTP) with hepatocyte-specific primers for albumin (5'-GAG AAG AAG TGC TGT GCT GCT GAA GG-3' and 5'- TCA GAG TGG AAG GTG AAG GTG-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 TCG CCT GGT CAC TG-3' and 5'- GCC ATC TTG GTC TGC GTC AT-3'), PCR cycles were as follows: initial denaturation at 95 °C for 15 min, followed by 35 cycles at 94 °C for 45 s, at 55 °C for 1 min, at 72 °C for 45 s, and final extension at 72 °C 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

| First antibody | Dilution | Second antibody | Dilution | Chromogen |
|----------------|----------|-----------------|----------|-----------|
| OV6 (mouse IgG) | 1:100 | Goat anti-mouse IgG (HRP or FITC) | 1:150 | DAB (brown) |
| CK19(mouse IgG) | 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) | 1:400 | Goat anti-mouse IgM (HRP) | 1:200 | DAB (brown) |
| C-kit (rabbit IgG) | 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.

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 °C with 50 mL/L CO2. 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.

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 nonparenchymal cell portion by low-speed centrifugation (50 r/min×1 min). About one third of the total liver cells were nonparenchymal cells.

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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 occurred 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. The control rat liver showed complete lobular structure without oval cell proliferation (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 indicate 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 positive 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).
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 positive 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.

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 immunohistochemical staining for OV6 (brown) and albumin (red). C: Staining for cytokeratin19(CK19). Ductular lumen face and oval cells were positive. Arrows point at CK19-positive cells. D: Double immunohistochemical staining for CK19 (brown) and albumin (red). Arrows point to oval cells with low 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.

Figure 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 colony on d 7. Most cells had their nuclei stained with BrdU (arrow). Though they came from one precursor, 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 colony with albumin (dark blue) and CK19 (brown). Some cells were stained with both markers (Arrows) and the others were stained only one marker. (×400).

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 alterative 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 hepatectomy. Immunohistochemical staining showed that the oval cells expressed oval cell-specific marker OV6, cholangiocyte lineage marker CK19 and hepatocyte 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\cite{23}. Since the oval cells were generated by the proliferation of terminal bile ducts, they formed structures representing an extension of the canals of Hering\cite{24}, 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\cite{26,27}. Chen et al.\cite{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\cite{8-11}. After bone marrow transplantation, a proportion of the regenerated hepatic cells was shown to be donor-derived\cite{29}. Intravenous injection of adult bone marrow cells (c-kit\textsuperscript{hi}Thy\textsuperscript{low}Lin\textsuperscript{−}Sca\textsuperscript{+}) into the FAH\textsuperscript{−}−/− mouse, an animal model of tyrosinemia type I, rescued the mouse and restored the biochemical function of its liver\cite{30}. Crosby et al.\cite{14} reported that there were c-kit\textsuperscript{+} cells in the perportal tract surrounding bile ducts, which coexpressed CD19. After cultured in biliary cell growth medium, the isolated c-kit positive cells could form colony with the properties of cholangiocytes\cite{15}. We also found that oval cells in the perportal area in 2-AAF/PD 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 extrathepatic origin of hepatic progenitors.

Suzuki et al.\cite{31} recently reported that CD49f\textsuperscript{-}c-Met\textsuperscript{−} c-KIT CD45 TER199 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\cite{32}. 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 animal, respectively\cite{33,34,35}. Tatematsu et al.\cite{36} reported that transfused oval cells in 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\textsuperscript{+} or CD34\textsuperscript{+} cells by Crosby et al. could only differentiate along cholangiocyte lineage\cite{37}. 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/PD rat liver. The properties of proliferation and differentiation in vivo are still to be studied by cell transplantation.

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