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Expression of neural-glial cell markers in cells from non-parenchymal progenitors of postnatal liver

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ABSTRACT

Increasing evidence provides support that postnatal hepatic stem/progenitor cells (HSPCs) have capability to express multi-tissue lineage markers in response to specific condition. Here, we reported that hepatic non-parenchymal progenitors, monkey liver epithelial progenitor cells (mLEPCs), acquired characteristics of neural-glial cell after a two-step induction. The E-cad+CD34-CD45-CD90- sorted adult mLEPCs have capacity of clonal growth and represent unique antigenic profiles. They generated into AFP and CK19 double positive cells in three Matrigels systems. After a two-step induction by growth factors stimulation, some cells converted to neural-glial like cells. Immunostaining showed that differentiated cells had acquired the expression of neural-glial cell markers (GFAP, A2B5, O4 and MBP) but lost the expression of epithelial cell markers (E-cad and CKs). RT-PCR further revealed that differentiated cells had acquired of expression of mRNA PDGFR-A, PDGFR-B, PLP, GFAP and MAG. Our results firstly demonstrated that at least a subpopulation of adult of mLEPCs expressed typical markers of neural-glial cells in specific environment condition.

KEYWORDS

Hepatic stem/progenitor cells;
Differentiation;
Glial cells;
Rhesus monkey.

INTRODUCTION

Hepatic stem/progenitor cells (HSPCs) are anticipated to become a potentially useful cell source for various cell therapies, bio-artificial livers, and drug discovery support systems. In addition, substantial literatures described the HSPCs had the potential to express multi-tissue lineage markers, including pancreatic cells[1], intestinal cells[2], cardiac cells[3,4], chondrocytes and osteoblatst[5,6], hinting that the HSPCs show plasticity with differentiation potential into hepatic an non-hepatic lineages. Furthermore, murine liver stem cells display some evidence of plasticity with differentiation into nerve cells when grown in suspension to form cellular spheres/bodies[7] or transplatation to the neonatal mouse brain[8]. The neural differentiation of HSPCs by monolayer induction is of interest, because it may provide information on the mechanisms of neural differentiation.

Previously, we reported the isolation and characterization of rhesus monkey liver epithelial progenitor cells (mLEPCs) from both adult and newborn liver[9,10]. On the base of above researches, we demonstrated that adult mLEPCs acquired the expression of hepatic stellate cells specific protein when co-cultured with monkey ear skin fibroblasts[11] and converted into myofibroblasts-like cells by TGFâ induction[12]. Here,

we reported that purified non-parenchymal progenitors from postnatal monkey liver expressed neural-glial cells specific markers after a two-step monolayer induction.

EXPERIMENTAL SECTION

Cell isolation and culture

Livers were obtained from healthy adult rhesus monkeys that were euthanized for industrial vaccine production; these animals had never been used in other experiments. Liver tissue was processed as previously described[9,10]. Briefly, about 5 g of liver tissue obtained from each animal was cut into small pieces and incubated in DMEM containing 0.2% collagenase (Invitrogen, Fremont, CA) and 0.07% DNAse for 30 min at 37°C in a shaking water bath. The cells in the supernatant fluid were collected, filtered through sterile gauze and centrifuged at 200 × g for 5 min. To get non-parenchyamal cells, the cell pellet was resuspended in 30 ml DMEM and centrifuged for 1 min at 50 × g 3 times; each time, cells in the supernatant were collected by centrifugation at 200 × g for 5 min. To exclude hepatic stellate cells and fibroblasts, cell aggregates were prepared in the presence of DMEM and 10% FBS. Single aggregates were seeded on 96-well plates (one single aggregate each well)(Invitrogen, Fremont, CA) coated on rat tail collagen gels in medium DMEM/F12 (Invitrogen, Fremont, CA) supplemented with 10% FBS (Hyclone, Logan, UT), 50 ng/mL epidermal growth factor (EGF, Sigma, St Louis, MO), 10ng/ml hepatocyte growth factor (HGF, Chemicon, Temecula, CA)10 mmol/L nicotinamide (Sigma, Steinheim, Germany), 1× ITS (Invitrogen, Fremont, CA), 100 U/ml penicillin(Sigma, Louis, MO), 100 µg/ml streptomycin (Sigma, Louis, MO). All cultures were maintained at 37°C in a 5% CO_2 incubator with medium changed every other day.

FACS analysis and sorting of cells

Polygonal epithelial cells expanded from aggregates were suspended at a concentration of 0.5-1 × 10^6 in PBS and incubated for 30 min at 4°C with the following antibodies: CD34-FITC, CD45, CD90 and E-cad-PE. PE- or FITC- conjugated secondary antibodies were used to detect unconjugated primary antibodies. The corresponding isotypes were used for evaluation of non-specific binding of monoclonal antibodies. The cells were then washed and suspended in Isoton for reading with a Beckham Coulter flow cytometer. The sorted cells were seeded on rat tail collagen gels coated 4-well plate at a density of 100 cells/well. For subpassaging, confluent cultures were split 1:1.5~1:2 using half-strength trypsin-EDTA (Invitrogen, Fremont, CA) for 10-30 minutes at 37°C.

In vitro differentiation of mLEPCs

For hepatic differentiation: Cells at less that 10 passages were cultured 7 days in 3-D Matrigel systems (A total of 0.2 mL of Matrigel was placed onto one well/4-well plate, permitted to set for 1 hour at 37°C) in DMED/F12 supplemented with 10%FBS, 20 ng /ml HGF, 1×ITS and 2mM glutamine.

For glial-like differentiation: A two-step induction method was employed starting with cells at less than 10 passages plated on gelatin coated dish at a cell density of 3 × 10^3 per cm^2 in DMEM medium supplement with supplemented with 2% FBS, 1µM dexamethasone (Sigma, St Louis, MO), 100 mg/ml 3-isobutyl-1-methylxanthine (IBMX) (Sigma, St Louis, MO), 50 mM indomethacin (Sigma, St Louis, MO) for 2 weeks (step 1), and then to DMEM medium supplemented with 2% FBS, 10ng/ml PDGF-AB (R & D Systems, Minneapolis, MN), 10ng/ml TH (Sigma, St Louis, MO), and 100 mg/ml 3-isobutyl-1-methylxanthine for 1 week (step 2). Medium was changed twice weekly.

Immunocytochemistry (ICC)

Cells were fixed with 4% paraformaldehyde in PBS for 10 min and washed with PBS three times, followed by permeabilization with 0.2% Triton ×-100 for 10 min and blockage with 4% goat serum for 30 min at 25°C. Subsequently, the cells were incubated with the primary antibodies (see TABLE 1) in staining solution (PBS containing 4% goat serum) for 40 min at 37°C, and then incubated with the appropriate Texas red, PE or FITC- conjugated secondary antibody in staining solution for 30 min at 37°C. The negative control was incubated in the staining solution without primary antibodies.

Reverse transcription-polymerase chain reaction (RT-PCR)

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TABLE 1 : Primary antibodies

Primaryantibody	Species	Manufacturer	Dilution
AFP	Mouse MAb IgG1	Sigma	1:200
A2B5	Mouse MAb IgG1	Santa Cruz	1:50
CK7	Mouse MAb IgG1	DAKO	1:100
CK8	Mouse MAb IgM	DAKO	Ready to use
CK18	Mouse MAb IgG1	Sigma	1:200
CK19	Mouse MAb IgG1	DAKO	1:100
E-cad	Mouse MAb IgG1	DAKO	1:100
GFAP	Mouse MAb IgG	DAKO	1:200
MBP	Rabbit MAb IgG	DAKO	1:200
O4	Mouse MAb IgM	Chemicon	1:150

Total RNA was isolated with Trizol reagent (Invitrogen, Calsbad, CA) according to the manufacturer's protocols. Reverse transcription was carried out with approximately 1 μg of total RNA in 20 μL of 1 x master mix (1 × reverse transcription buffer, 0.5 mol/L dNTPs, 50 pmol of oligo(dT) primer, 20 U of RNase inhibitor and 5 U of reverse transcriptase) at 42°C for one hour. For PCR, 1 μL of RT products was added to 1 × PCR master mix (1 × PCR reaction buffer, 1.5 m mol/L of MgCl2, 0.5 m mol/L of dNTPs, 0.4 μmol/L of forward primer, and 0.4 μmol/L of reverse primer (see supplemental TABLE 1), 1.25 U of Taq DNA polymerase in a 25 μL final volume and amplified by 25-35 cycles of PCR (95°C 30 sec; 52-60°C 30 sec; 72°C; 30 sec) followed by a final extension at 72°C for 5 min. All reagents in RT-PCR were purchased from Takara (Takara, Dalian, China) unless otherwise mentioned. Products of PCR were separated on a 2% agarose gel and stained with ethidium bromide. The primer sequence for *GAPDH* is 5′-*TGAAGGTCGGAGTCAACGGA* -3′/5′-*TGGTGCAGGAGGCATTGCTG* -3′; for *GFAP* is 5′- *AGTCCAAGCAGGAGCACAAG* -3′/5′-*CAAAGGCACAGTTCCCAGATA* -3′; for *PDGFRa* is 5′-*CCAAGCCTGACCACGCTAC* -3′/5′-*TCATCCAGACCACCCTCCC* -3′; for *PDGFRb* is 5′- *CTCACCATCATCTCCCTTATC* -3′/5′-*GTCTCCGTAGCGGCAGTA* -3′; for *PLP* is 5′-*ATTCTGTGGCTGTGGACATG* -3′/5′-*GGACGGCAAAGTTGTAAGTG* -3′; for *MAG* is 5′-*CCACCGCCTTCAACCTGTCT* -3′/5′-*CCTGCGTGTCTGGGTAATGT* -3′.

Statistical analysis

All data were expressed as Standard Error. Statistical comparisons were performed using Student's t-test ($P < 0.05$ was considered statistically significant).

RESULTS AND DISCUSSION

Clonal culture, phenotype and hepatic differentiation of adult mLEPCs

In order to get purified non-parenchymal progenitors with high growth capacity, multiple methods were applied as previously described[9]. Firstly, freshed digested hepatic cells were centrifuged for 1 min at 50×g 3 times to get non-parechymal cells and eliminate parechymal cells i.e. mature hepatocytes. Secondly, collected non-parenchyamal cells were treated by Ca^{2+}-containing medium to generate cell aggregate, in which hepatic stellate cells, fibroblasts and Kuffer cells could not form cell aggregates because of lack of cadherin-dependent cell-cell adhesion. Attached cell aggregates represented either of two distinct epithelial cell types: vascular endothelial cell aggregates and polygonal epithelial cells. Vascular endothelial cells expressing vimentin and brachyury were disappeared within 5 days in our culture system, while polygonal epithelial cells expressing E-cadherin had robust proliferation. Finally, after expansion of polygonal cells in cell aggregate, FACS (with E-cad+ CD34-CD45-CD90-) was then employed to yield a homogeneous epithelial progenitor populations (99.8%) (Figure 1).

For assessment of clonal growth, sorted polygonal epithelial were inoculated at a low density (100 cells/well) on rat tail collagen gels coated 4-well plates. Small colonies were formed within 4-5 days, moderate colonies were formed within 7-8 days and large colonies containing thousands of cells were formed by 10-12 days (Figure 2). The average double time of the cells is~20 hours. The epithelial cells in large colonies were characterized as being less than 9±1 μm in diameter, and having scanty cytoplasm, prominent round or oval-shaped nuclei and a high nuclear to cytoplasm ratio.

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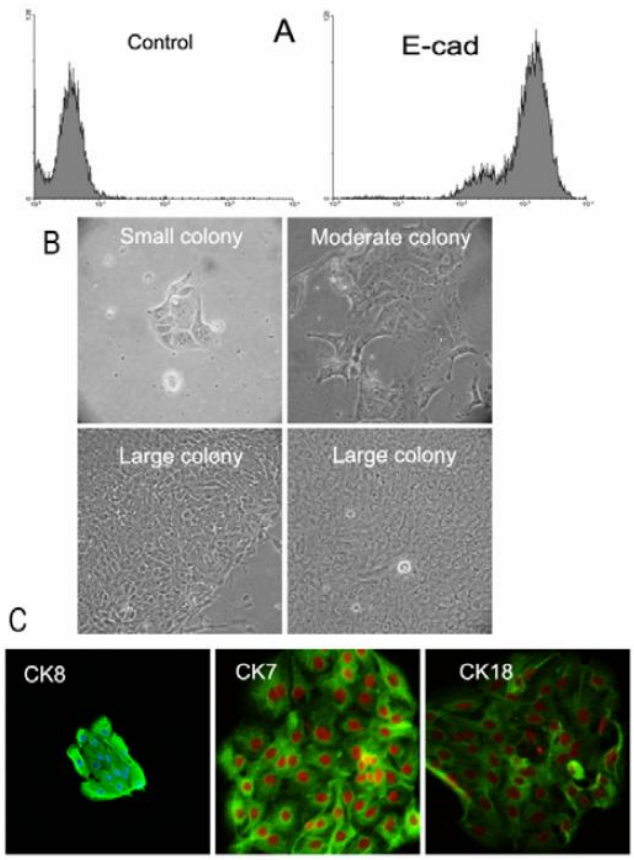

Figure 1 : Characterization of mLEPCs; A) FACS analysis of adult mLEPCs. Histograms for E-cad are presented in direct comparison to their isotype controls. B) Sorted polygonal epithelial were inoculated at a low density to form cell colonies. C) The adult mLEPCs were positive for epithelial markers CK8 (green) CK7 (green) and CK18 (green), Nuclear DNA was counterstained with PI (red) or Hoechst (blue)

The morphology of such epithelial colonies was similar to that of the cultured bipotent HSPCs in postnatal mouse liver[13,14].

Immunofluorescence analysis using antibodies revealed that nearly 100% of colonies consisted of cells positive for Cytokeratin 7 (CK7), CK8 and CK18 (Figure 1.C), but negative forα-feteoprotein (AFP) and albumin (ALB). In some circumstance, epithelial cells in colonies faintly expressed CK19. FACS analysis of cell surface molecules showed that adult mLEPCs were homogenously positive for CD44 and CD73 (data not shown). Thus, the protein expression pattern of normal HSPCs from adult liver were different from hepatoblasts or oval cells, which were known for highly expressed AFP, ALB and CK19. In fact, they were largely consistent with those of hepatic progenitors from normal adult liver in recently reported literature[13,15]. Adult mLEPCs were cultured in 3-D Matrigels systems for testing their capability to differentiate into hepatic lineages in vitro. After induction for 14 days, differentiated cells were found to co-express the protein of AFP and CK19 (Figure 2). Quantitation of positive-scoring cells (as a percentage of total number of positive cells in multiple fields of Hoechst positive cells) showed that more than 70% of cultured cells were AFP positive. It is well known that AFP is the specific markers for immature hepatocytes, while CK19 is expressed in the mature cholangiocytes. By periodic acid- Schiff (PAS) staining, the differentiated cells could store glycogens (Figure 2). This observation indicated that mLEPCs from normal adult liver were the progenitors of hepatocytes.

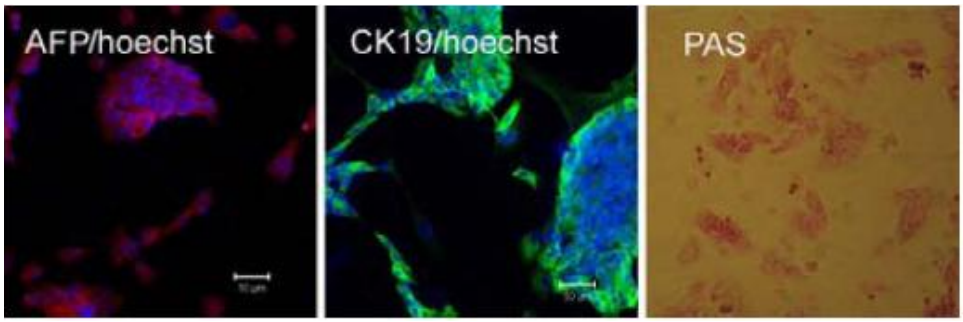

Figure 2 : Differetiation of mLEPCs into hepatic cells; The adult mLEPCs differentiated into AFP positive hepatocytes (red), CK19 positive cholangiocytes (green) and PAS positive hepatocytes

Expression of neural-glial cell markers in mLEPCs-derived cells

The role of IBMX, PDGF and TH in neural-glial cells differentiation has been detailly described in many literatures[8,16-18]. The use of chemical reagents such as IBMX, dbcAMP and BHA has indeed induced neural morphological acquisition in HSPCs, as has been reported in the MSCs after treated with similar induction protocols. To evaluate the ability of mLEPCs to differentiate in the neural-glial cell lineage, the cells were exposed to medium consisting of IBMX, PDGF-AB and

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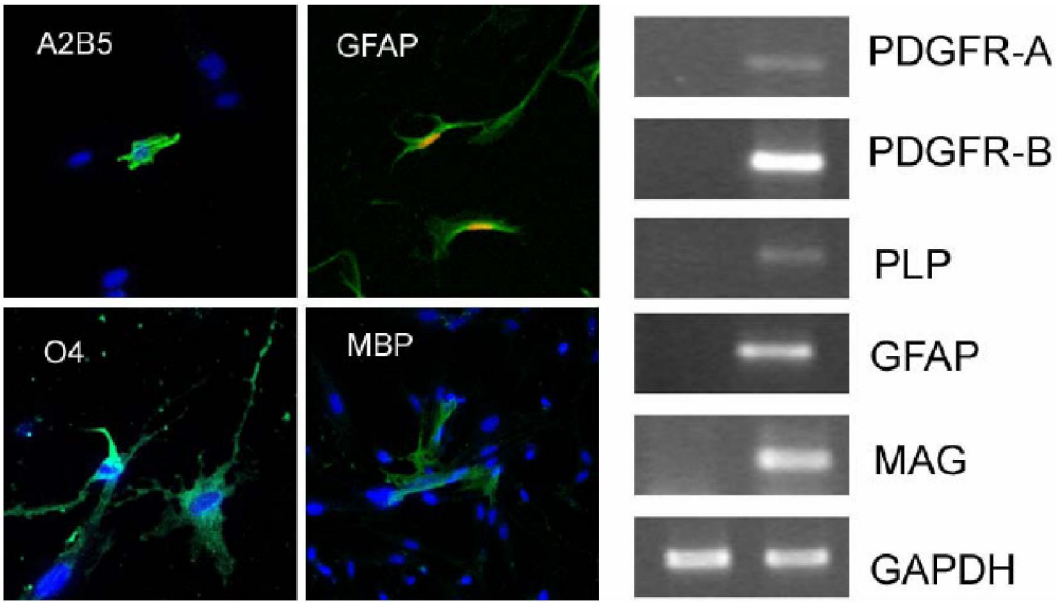

Figure 3 : Expression of neural-glial cell markers in induced mLEPCs; The mLEPCs derive cells were positive for neural-glial cell markers by expression protein of A2B5, GFAP, O4 and MBP and genes of PDGFR-A, PDGFR-B, PLP, GFAP and MAG

TH by a two-step induction method. During this time, some cells slowly transitioned from an epithelial morphology to a fibroblast-like shape or acquired a star morphology. These differentiated cells were then fixed and stained for neural markers. Some cells (about 10%) were strongly positive for glial cells typical markers such as A2B5, GFAP, O4 and MBP, which were not detected in mLEPCs. As expected, RT-PCR reveal that a set of glial cell genes including PDGFR-A, PDGFR-B, PLP, GFAP as well as MAG, were detected in these differentiated cells but not in untreated mLEPCs (Figure 3). However, the expression of neuron markers β-tubulin III or NCAM were not presented in these differentiated cells, indicating neuron were not induced.

In combination with cell morphology, mRNA and protein expression analysis, our data showed that glial-like cells were differentiated from adult mLEPCs. Similarly, previously report indicated that murine resident liver stem cells could express typical marker of neuronal and glial cells after exposure to neurogenic condition[7] or in IBMX/dcAMP containing medium[8]. However, results indicated that the neural-like induction response to such 'chemical induction' exhibited in the mesenchymal or hepatic stem cells is the result of cell stress, rather than true neuronalization. Although the efficiency of differentiation achieved was relatively low (~10%), we believe that the observed glial cell behavior represents a trait of plasticity attributed to a subpopulation of HSPCs.

CONCLUSION

In conclusion, we firstly demonstrated that at least a subpopulation of adult E-cad positive mLEPCs-derived cells expressed typical markers of glial cell in response to specific environment condition, which would help us to further understanding the plasticity of stem cell.

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