Mesenchymal-Epithelial Transformation of Ito Cells *In Vitro*

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Translated from *Kletochnye Tekhnologii v Biologii i Medicine*, No. 3, pp. 150-153, August, 2006
Original article submitted March 6, 2006

Cultured pure population of Ito cells isolated from adult rat liver expressed epithelial markers cytokeratin-8, α-fetoprotein, and γ-glutamyl transpeptidase after forming a dense monolayer. Mesenchymal-epithelial transformation of these cells is possible, which suggests them as candidates of hepatic stem cells.

Key Words: *Ito cells; stem cells; liver; ontogenesis; regeneration*

Mature hepatocytes can participate in liver regeneration [2]. It was persuasively demonstrated that hemopoietic stem cells can restore the parenchyma of the damaged liver [19]. The problem of liver stem cells is actively disputed in recent years. Many scientists consider that this role can be played by oval cells appearing in the liver in some models of regeneration (toxic hepatic injury in rats or partial hepatectomy with simultaneous blocking of hepatocyte proliferation) [16]. However, despite numerous studies, regional hepatic stem cell is not identified.

In many cases of liver regeneration the count of Ito cells and their proliferation increase at the initial stages (at the peak of hepatocyte proliferative activity) irrespective of participation of oval cells in this process [13,14].

High density of Ito cells was also detected during liver development; their marker (desmin) is also expressed in hepatoblasts at the early stages [8]. Hence, hepatic epithelial cells can produce phenotypical markers of mesenchymal Ito cells during prenatal development. In order to clear out whether Ito cells can express epithelial markers, we studied the dynamics of phenotypical variability of Ito cells during culturing of their pure population isolated from the liver of adult rats.

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MATERIALS AND METHODS

The cells were isolated from the liver of male rats (300-400 g) under nembutal narcosis (0.01 ml/100 g). Ito cell fraction was obtained by collagenase-pronase treatment of the liver [4,9] in Nikodense density gradient [1].

The cells were cultured in DMEM (25×10^6 cell/ml) with 10% FCS, antibiotics, and antimycotics in 24-well plates with slides on the bottom of the wells. The plates were placed in a humid CO_2 incubator at 5% CO_2 and 37°C. The medium was replaced for the first time after 24 h, then every other day. On day 1 of culturing the cytoplasm of about 90% cells contained lipid drops, which is typical of Ito cells. During replacement of culture medium some slides were fixed in acetone (for immunohistochemical tests). Cell culturing was carried out until the formation of a dense uniform monolayer.

After that the cells were treated with trypsin in buffered saline by the standard method, reinoculated at the same density as newly isolated cells, and cultured under the same conditions.

Immunocytochemical staining was carried out by the indirect immunoperoxidase and streptavidin-biotin methods using visualization systems and commercial monoclonal antibodies to desmin, α-smooth-muscle actin (A-SMA), collagens I, III, and IV, laminin, cytokeratin-8, and α-fetoprotein

0007-4888/06/1421-0133

(DAKO, Novocastra). γ-Glutamyl transpeptidase (GGT) was detected histochemically [15].

RESULTS

Fresh isolated cells inoculated in culture were characterized by typical morphology of Ito cells (cells with processes and lipid drops in the cytoplasm; Fig. 1, *a*). Cells adhering to the glass were stained with antibodies to vimentin and desmin. No artificial microenvironment was created for the cells; growth factors were not added into the medium. By the end of week 1 cultured cells were stained with antibodies to collagens I, III, and IV and laminin.

Cell density and intensity of staining with antibodies to desmin increased during the 1st week of culturing (Fig. 1, *b*). Some cells located at the periphery contained stress fibers and expressed A-SMA. The number of such cells (myofibroblasts) gradually increased. However, with increasing cell density and formation of the monolayer myofibroblasts could be seen only at sites free from the bulk of cells.

The cells were not re-inoculated after attaining confluence (when cell count increases so that the majority of cells contact with the neighbors). By the end of week 2 a cell monolayer formed and retraction of cytoplasmatic processes was observed. Clusters of cells containing GGT were detected during this period (Fig. 1, *c*). During week 3 of culturing myofibroblasts disappeared as the monolayer density increased. Solitary cells were found only at free sites of slides, not occupied by the monolayer. The process of monolayer packing, when the cell is surrounded from all sides and contacts with its neighbors, was associated with not only retraction of cytoplasmatic processes, but also desmin repression. After 3 weeks of culturing, when the monolayer morphologically looked like an epithelial cell monolayer, but not a fibroblast culture, the cells were treated with trypsin and reinoculated to new slides.

Reinoculated cells acquired typical morphology of Ito cells during the first 24 h after alteration of the microenvironment: they formed numerous processes and contained lipid drops in the cytoplasm (Fig. 2, *a*). Some cells were binuclear, which could result from the beginning of mitosis. However, it is also possible that some of them were polyploid during reinoculation.

During week 1 of culturing reinoculated cells started expressing desmin (Fig. 2, *b*). Similarly as in passage 1 cells, signs of synthesis of intercellular matrix macromolecules were detected in their cytoplasm during the first two weeks of culturing: they were stained with antibodies to laminin and collagens III and IV (Fig. 2, *c*).

As the density of cells increased, their morphology changed: retraction of processes was paralleled by the formation of a compact layer resembling an epithelial one (Fig. 3, *a*). On day 9, despite the beginning of monolayer formation, GGT was detected in the cytoplasm of only solitary cells, while by the end of week 3 virtually all cells in the culture were stained for GGT (Fig. 3, *b*). Moreover, by the end of week 3 of culturing some cells were stained with antibodies to α-fetoprotein. Cytokeratin-8 appeared in the cytoplasm of larger cells morphologically resembling hepatocytes (Fig. 3, *c*).

Hence, Ito cells are capable of mesenchymal-epithelial transformation and express hepatocyte

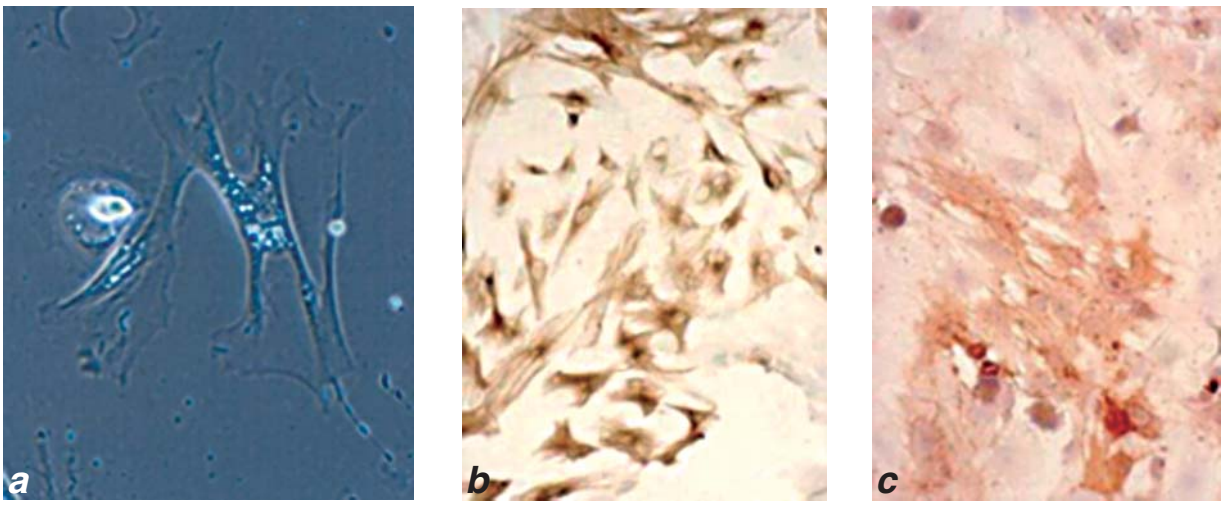

Fig. 1. Cultured Ito cells, passage 1. *a*) day 1 of culturing, phase contrast, ×400; *b*) desmin expression in Ito cells (day 3 of culturing), ×200; *c*) expression of γ-glutamyl transpeptidase in Ito cells (day 12 of culturing), ×200.

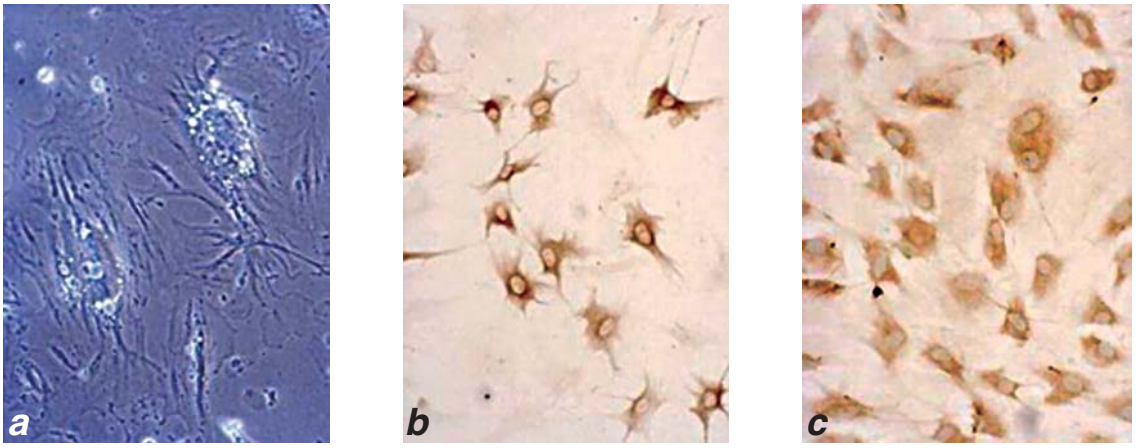

Fig. 2. Ito cells after trypsinization and reinoculation onto new slides (passage 2). *a*) day 1 of culturing: phase contrast, ×400; *b*) day 2 of culturing: desmin expression, ×200; *c*) day 8 of culturing: expression of collagen III, ×200.

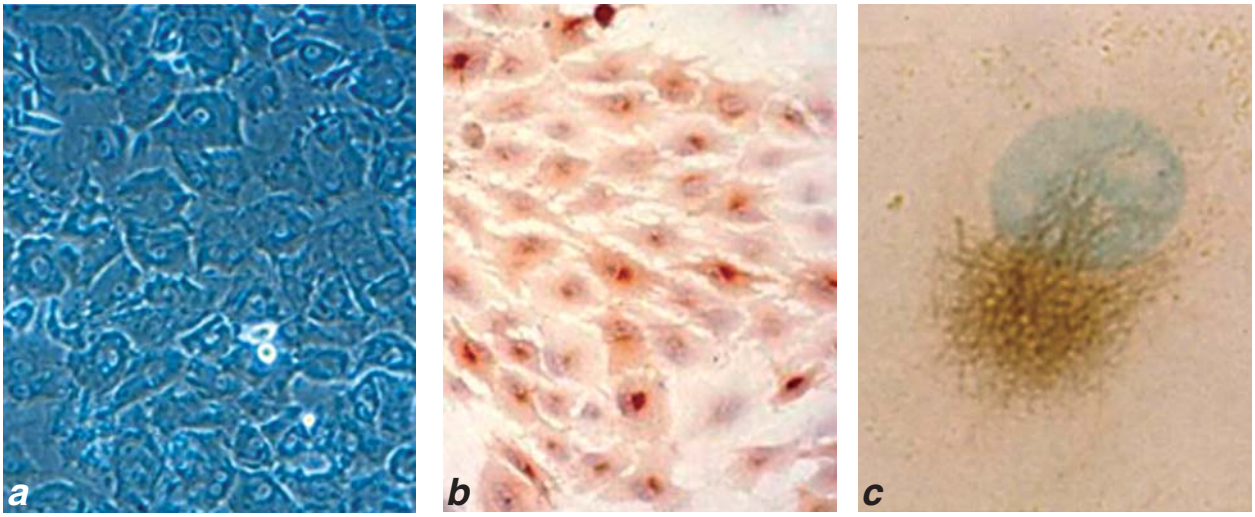

Fig. 3. Morphology of compact monolayer cells (passage 2). *a*) day 26 of culturing: phase contrast, ×200; *b*) day 21 of culturing: expression of γ-glutamyl transpeptidase, ×200; *c*) day 24 of culturing: expression of cytokeratin-8, ×1000.

and oval cell markers (GGT, cytokeratin-8, and α-fetoprotein). Expression of epithelial markers in a compact monolayer of Ito cells in our experiments can be attributed to possible contamination of the culture with hypothetical hepatic stem cells (epithelial progenitor cells). However, the most possible explanation is that Ito cells under certain conditions are capable of the mesenchymal-epithelial transformation. Recent reports confirm our hypothesis. It was shown, for example, that the onset of hepatic stage of hemopoiesis is associated with high density of Ito cells in the liver in general and in hemopoietic foci [8], Ito cells in hemopoietic foci possessing a mixed epithelial/mesenchymal phenotype [6]. It was also shown that bone marrow (mesenchymal) cells restore damaged liver [3]. After allogenic transplantation of bone marrow cells from transgenic (GFP) mice donor cells migrated into the recipient liver and acquired the phenotype of Ito cells [5]. The possibility of hepatocyte formation from mesenchymal cells isolated from human adipose tissue *in vitro* and *in vivo* [17] and from mesenchymal stem cells *in vitro* was demonstrated [10]. Simultaneous expression of cytokeratins and CD34 (a marker of hemopoietic stem cells) [18] and epithelial markers cytokeratin-18, cytokeratin-19, and E-cadherin [11,12] was detected in Ito cells from human fetal liver. Entodermal origin of Ito cells was hypothesized, and the expression of mesenchymal markers in these cells was assumed to be a result of the mesenchymal-epithelial transformation [11,12]. Fetal origination of Ito cells remains not studied [7], but they are known to play the key role in liver development and regeneration. The possibility of mesenchymal-epithelial transformation of Ito cells detected in our study is one

more fact pointing with higher probability to this cell type as a rat liver stem cell.

The study was supported by the Russian Foundation for Basic Research (grant No. 04-04-49164).

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