Liver Repopulation Capability of Hepatic Stem/Progenitor Cells Isolated by Flow Cytometric Cell Sorting （フローサイトメトリーを用いて分離した肝幹細胞/前駆細胞の組織再構築能）

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Summary

Background

There is an increasing shortage of donor livers for clinical therapies since liver organ transplants are the only available and efficient treatment for the patients with end-stage liver failure. Therefore, there is a pressing need to develop alternative therapeutics. Liver cell transplantation may be one of the effective therapeutic approaches instead of liver organ transplantation in clinic. Based on recent studies, hepatic stem cells may be therapeutically useful for the treatment of a variety of liver diseases, including acute liver failure and a number of liver genetic diseases. Stem cells might prove to be ideal vehicles for delivering therapeutic genes to the liver. Although the existence of a liver stem cell was controversial for many decades, it is now generally accepted that the liver contains cells with stem-like properties and these cells can be activated to proliferate and differentiate into mature hepatic epithelial phenotypes (hepatocytes and cholangiocytes) under certain pathophysiological circumstances. Because of the lack of specific markers for hepatic stem cells, the origin of oval cells and their precise location within the liver, especially within the normal liver have remained obscure.

Since fetal hepatocytes contain highly proliferative, bipotential epithelial cells and are also easily to be collected under non-pathological

conditions, successful separations and enrichments with flow cytometry for hepatic stem cells or progenitors from fetal livers have been reported recently. Similar to the mentioned above, specific selective markers for fetal hepatic stem cell have not been identified. The most convincing proof of bipotentiality will be provided by *in vivo* cell transplantation studies because this microenvironment will support differentiation of hepatoblasts along both hepatocyte and biliary lineages. On the other hand, not only bipotent nature, but also clonality of sorted liver stem cells or progenitors needs to be confirmed by *in vivo* transplantation studies. Although the transplantation for non-fractionated fetal liver progenitor cells has met partial demands, according to our knowledge, until now there is few reports on *in vivo* for the hepatic stem cells or progenitors that isolated by the means of fluorescence-activated cell sorting (FACS).

We here focus on *in vivo* studies to compare FACS fractionated fetal liver cells of rat for their proliferated capability, clonality and bipotency. ICAM-1^{+}RT1$A^{-/low}$CD$45^{-}EC^{-}$ cells in rat liver cells expressed much higher regenerative capability in recipient livers than any other fractions. The characterization for the fraction ICAM-1^{+}RT1$A^{-/low}$CD$45^{-}EC^{-}$ involved *in vitro* also provided us the evidences that it possessed the properties of hepatic stem cells.

Essential Methods

Cell transplantation model: The cannicular enzyme Dipeptidyl Peptidase IV (DPPIV) can be expressed in wild type animals except in one substrain of inbred Fischer rat strains. DPPIV is highly expressed in liver epithelial cells, hepatocytes and bile duct epithelial cells. In this study, DPPIV deficient Fischer rat was treated with retrorsine and two-third partial hepatectomy, transplanted $DPPIV^{+}$ wild type hepatocytes of normal Fischer rat are then determined by enzyme histochemistry or immunohistochemistry in the recipient of $DPPIV^{-}$ mutant rat. Here, treated with retrorsine, a DNA-alkylating agent that disrupts cell cycle progression in hepatocytes, so that prevented the recipient hepatocytes from proliferating. When retrorsine treated rats are subjected to partial hepatectomy, endogenous hepatocytes are unable to proliferate but wild-type transplanted cells selectively proliferate and repopulate the liver.

Flow cytometric analysis and sorting: After immunostained with monoclonal antibodies for intercellular adhesion molecule 1 (ICAM-1), classical major histocompatibility class I (MHC-I, Fischer rat as $RT1A^{l}$), hepatocyte growth factor receptor (c-Met), leukocyte common antigen (CD45) and rat erythroid cells (EC), we used five-dimensional flow cytometry (FACSVantage) to profile antigenic features and sort the subpopulations of ED13.5 to ED15.5 fetal liver cells from normal Fischer rats.

***In vitro* and *in vivo*:** Fractionated and sorted cells by flow cytometry were cultured on collagen type IV coated dishes for 12-day at extreme low density

(200cells/cm^2) in DMEM/F12 supplemented with FCS and growth factors for the analyses of clonogenic potential and bipotential capabilities. And *in vivo* study, freshly isolated fetal liver cells by FACS were infused into treated recipient livers via portal vein for 3-month and quantitative detection for repopulation rates, repopulated colony numbers and others relevant to donor cells in recipients was computed and analyzed.

Cell cycle analysis: BrdU short pulse labeled fetal liver cells were sorted by FACSVantage and after PI treatment, analyzed by FACSCalibur for cell cycle distribution and nuclei ploidy by DNA analysis.

Results

By FACS analysis, we could obtain an antigenic profile of ICAM-1 and RT1A^1 for early developing stage of fetal liver cells. There is a subpopulation separated clearly from main population expressed as ICAM-1$^+$RT1A$^{-/low}$CD45$^-$EC$^-$ proportioned about 2.7% in ED14.5 fetus. This subpopulation interlapped with HGF receptor (c-Met$^+$) about 23% and enriched about 11-fold.

By *in vitro* for clonogenic potential and bipotential, comparing with main population or non-fractionated liver cells, the subpopulation ICAM-1$^+$RT1A$^{-/low}$CD45$^-$EC$^-$ more frequently produced H-CFU-C (Hepatic Colony-Forming-Unit in Culture) than any other cell groups. We defined a count only when the colony contained more than 100 cells. ICAM-1$^+$RT1A$^-$CD45$^-$EC$^-$ and ICAM-1$^+$RT1A^{low}CD45$^-$EC$^-$ cells could produce H-CFU-C in number as about 8.5 and 4.8 respectively relative to per 2x10^3 inoculated cells, whereas CD45$^+$EC$^+$ (hematopoietic cells), non-fractionated and main population cells rarely formed any H-CFU-C. The fractionated cells of ICAM-1$^+$RT1A$^{-/ow}$CD45$^-$EC$^-$ could form relative a large number of H-CFU-C in every experiment and the colony constituted by more than one thousand cells was observed frequently. Furthermore, the colony from a single cell in subpopulation of ICAM-1$^+$RT1A$^{-/ow}$CD45$^-$EC$^-$ could express hepatocyte marker, albumin and cholangiocyte marker, cytokeratin 19 simultaneously by immunohistochemistry.

By *in vivo* transplantation to investigate the regenerative potential of the subpopulation of ICAM-1$^+$RT1A$^{-/low}$CD45$^-$EC$^-$, fractionated and non-fractionated fetal liver cells, they were divided into eight groups to be compared. To compare the groups of ICAM-1$^+$RT1A$^-$CD45$^-$EC$^-$ and ICAM-1$^+$RT1A^{low}CD45$^-$EC with non-fractionated cells (10^{5}cell/recipient group), about 1/40 to 1/50 of infused cell numbers produced 6 to 10-fold repopulation rates and the total enrichments could be estimated between 200 to 500-fold. By the same comparison on repopulated colony numbers, there were 50 to 120-fold enrichments. Not only proliferation, but also the capability that differentiated into normal and functional liver cells from the donor hepatic stem/progenitor cells was confirmed on the repopulated colony in the recipient livers. Such as DPPIV and glycogen were detected on donor-derived cells, which had not or rare

expressed when transplanted.

By cell cycle and DNA content analysis, there were no any 4N DNA content cells (tetraploid) detected in subpopulation of ICAM-1^{+}RT1A$^{-/low}$CD45^{-}EC^{-}. In fraction ICAM-1^{+}RT1A$^{-/low}$CD45^{-}EC^{-}, there were about 2/3 cells in phase G0/G1 and about 1/3 in phase S, on the contrary, in non-fractionated liver cells about 1/4 in G0/G1 and about 3/4 in phase S. The analysis in short pulse labeling for BrdU showed that the fraction ICAM-1^{+}RT1A$^{-/low}$CD45^{-}EC^{-} had about 20% integration with BrdU and non-fractionated liver cells had about 65%. The relative low activity for division and high proportion in G0/G1 phase from the subpopulation implied that the subpopulation might possess the property of stem cells.

Discussion

As a more reliable and convincing approach, *in vivo* transplantation study, which is close to the real physiological conditions, has not been well documented systematically for the identification of the hepatic stem cells or progenitor cells isolated by flow cytometry. We focused on *in vivo* transplantation to examine enhanced proliferative activity, bipotential nature and repopulation capability in a normal liver environment, using the DPPIV deficient Fischer rat cell transplantation model system. In early developing rat livers (about ED13.5 to ED15.5), the antigenic profile for ICAM-1 and RT1A^{l} was separated into main-population and subpopulation by flow cytometry. The subpopulation cells expressed as ICAM-1^{+}RT1A$^{-/low}$CD45^{-}EC^{-} should be the candidates for hepatic stem cells, since it was verified *in vitro* as well as *in vivo* studies. ICAM-1^{+}RT1A$^{-/low}$CD45^{-}EC^{-} cells not only possess the clonogenic and bipotential capabilities *in vitro* comparing with the cells in main population of ICAM-1^{-}RT1A$^{+/-}$CD45^{-}EC^{-} and non-fractionated cells, but also contribute more to regeneration in recipient injured livers according to *in vivo* transplantation studies of quantitative detection for repopulation. DPPIV^{+} bile duct-like structure convinced us that the bipotent differential capability for ICAM-1^{+}RT1A$^{-/low}$CD45^{-}EC^{-} cells *in vivo*, which could differentiate into hepatic parenchyma as well as bile duct epithelium.

Cell cycle phase analysis emphasized and supported that ICAM-1^{+}RT1A$^{-/low}$CD45^{-}EC^{-} cells possessed the property of stem cells. The cell cycle profile of each population corresponds to their functional status: the most primitive stem cells are dormant (in G0 phase), the majority of self-renewing stem cells are in G1 phase and slowly cycling, and transit amplifying progenitors are rapidly cycling for effective expansion. Most normal resting cells are diploid, while normal proliferating cells exist at various levels of ploidy. The relative low activity for division and high proportion in G0/G1 phase implied that the subpopulation might possess the property of stem cells.

As one of putative hepatic stem cells the oval cell has attracted attentions for several decades, however, much of it is still unclear including whose progeny it is. Hepatoblasts derived from the foregut endoderma cells

differentiate into both hepatocytes and bile duct epithelial cells. Furthermore many results have supported the notion that oval cells have lineage options similar to those displayed by hepatoblasts in early stages of liver development such as CK19, albumin, AFP, BD1 (negative) and the oval cells also share a number of fetal isoforms of enzymes with hepatoblasts. One of the possibilities for the origin of oval cells is that in the developed liver the progeny of hepatoblasts exists as so called oval cells and keeps quiescent and small numbers in normal condition. ICAM-1^{+}RT$1A^{-/low}$$CD45^{-}EC^{-}$ cells as a subpopulation of fetal liver epithelial cells highly expressed stem cell properties *in vitro* and *in vivo* and existed but decreased in proportion from early stage of embryonic liver to neonate. Continually to explore the biological meaning of this subpopulation on adult liver will be valuable and informative.

MHC class I is one of the developmentally controlled antigens and performs a critical role in immune supervision. In fetal liver cell, it is easily to escape from the host immune system after transplantation due to low expression for MHC class I. A study for human fetal hepatocyte shows, there is also no MHC class I expression. Using the similar sorting technique, we may get the similar cell fraction with stem cell properties from human fetal and adult livers. Indeed, hepatic stem cells may be the cell of choice for clinical cell transplantation, gene therapies or bioartificial liver devices.

Conclusion

By flow cytometry, the isolated cells in subpopulation of ICAM-1^{+}RT1A (MHC-I)$^{-/low}$$CD45^{-}EC^{-}$ from early stage of embryonic Fischer rat possessed some properties of hepatic stem cells. They expressed clonogenic and bipotential capabilities *in vitro*. Furthermore, the stem cell properties were also reconfirmed *in vivo* transplantation study by the retrorsine/PH hepatocellular transplantation model. According to quantitative detection *in vivo*, the repopulation efficiency should be enriched no less than 200-fold comparing with non-fractionated fetal liver cells. Due to lack of the expression of MHC class I in human fetus similar to the animal used in this experiment, and if we could find out the same cell fraction in the human adult liver, there is possible to apply this approach to clinical research and therapies in the future.