BASIC AND TRANSLATIONAL—LIVER

Direct and Indirect Contribution of Human Embryonic Stem Cell–Derived Hepatocyte-Like Cells to Liver Repair in Mice

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BACKGROUND & AIMS: Many studies of embryonic stem cells have investigated direct cell replacement of damaged tissues, but little is known about how donor cell–derived signals affect host tissue regeneration. We investigated the direct and indirect roles of human embryonic stem cell–derived cells in liver repair in mice. **METHODS:** To promote the initial differentiation of human embryonic stem cells into mesendoderm, we activated the β-catenin signaling pathway with lithium; cells were then further differentiated into hepatocyte-like cells. The differentiated cells were purified by indocyanine green staining and laser microdissection and characterized by immunostaining, polymerase chain reaction, biochemical function, electron microscopy, and transplantation analyses. To investigate indirect effects of these cells, secreted proteins (secretomes) were analyzed by a label-free quantitative mass spectrometry. Carbon tetrachloride was used to induce acute liver injury in mice; cells or secreted proteins were administered by intrasplenic or intraperitoneal injection, respectively. **RESULTS:** The differentiated hepatocyte-like cells had multiple features of normal hepatocytes, engrafted efficiently into mice, and continued to have hepatic features; they promoted proliferation of host hepatocytes and revascularization of injured host liver tissues. Proteomic analysis identified proteins secreted from these cells that might promote host tissue repair. Injection of the secreted proteins into injured livers of mice promoted significant amounts of tissue regeneration without cell grafts. **CONCLUSIONS: Hepatocyte-like cells derived from human embryonic stem cells contribute to recovery of injured liver tissues in mice, not only by cell replacement but also by delivering trophic factors that support endogenous liver regeneration.**

Keywords: hES Cells; Hepatitis; Mouse Model; Stem Cell Therapy.

An important aspect of developing stem cell therapies is to determine the cellular basis for tissue repair by addressing the exact action mechanism of grafted donor cells. Although many studies have focused on the direct cell replacement of damaged tissues, potential contributions of donor cell–derived signals to host tissue regeneration are largely unknown. A series of recent findings have increased interest in the potential role of local or systemic soluble factors that support robust regeneration of injured and aged tissues.[1,2] Thus, determining how cell transplantation interacts with endogenous regenerative mechanisms is a fundamental question in achieving clinical success that has been less precisely defined.

Tissue regeneration requires highly coordinated events by host or grafted donor cells that lead to functional recovery of injured tissues. Unlike other organs in the body, the liver has a remarkable ability to restore considerable tissue loss in a relatively short time. This feature of the liver provides an ideal model system to study tissue repair mechanisms after transplant of stem cell–derived cells. Furthermore, clinical experiences with liver transplantation make liver diseases an attractive target for new therapies based on the ex vivo growth of stem cells.[3] An encouraging recent report shows that hepatocytes derived from human embryonic stem (ES) cells survive and express hepatic features in an animal model of liver disease.[4] This report and previous studies suggest that hepatocytes can be derived from human ES cells but do not provide detailed in vivo analysis that can distinguish the relative contributions of donor and host cells in the positive therapeutic outcome.[5–7] Using liver regeneration as a model system, here we show that donor cells derived from human ES cells actively contribute to tissue recovery not only by cell replacement but also by delivering trophic

Abbreviations used in this paper: Ad-luc, adenovirus expressing luciferase; BrdU, bromodeoxyuridine; DEX, dexamethasone; EB, embryoid body; ES, embryonic stem; GAS-6, growth arrest-specific 6; HGF, hepatocyte growth factor; HL, hepatocyte-like; HSC, hepatic stellate cell; ICG, indocyanine green; iPS, induced pluripotent stem; MFGE-8, milk fat globule-EGF factor 8; OSM, oncostatin M; Qsox1, quiescin Q6 sulfhydryl oxidase 1; VEGF, vascular endothelial growth factor.

0016-5085/$36.00
doi:10.1053/j.gastro.2011.11.030

factors that support endogenous regeneration of host injured tissues.

Materials and Methods

Differentiation and Transplantation of Human ES Cell–Derived Hepatocyte-Like Cells

Embryoid body (EB) formation was initiated by cultivating partially dissociated human ES and induced pluripotent stem (iPS) cell clumps. After the first 2 days of differentiation, EBs were grown with 10 mmol/L lithium chloride (Sigma, St. Louis, MO). After 2 days of induction, day 4 EBs were transferred into the original culture medium in the absence of lithium. After an additional 2 days, the lithium-treated EBs were plated onto collagen type I–coated culture dishes and allowed to differentiate for up to 20 days in the presence of 20 ng/mL hepatocyte growth factor (HGF; R&D Systems, Minneapolis, MN), 10 ng/mL oncostatin M (OSM), and 10^{-6} mol/L dexamethasone (DEX; Sigma). For in vivo study, BALB/c nude mice were treated with carbon tetrachloride (CCl_4) 1 day before transplant and were injected intrasplenically with 2.0×10^6 cells. All experimental procedures involving human ES cells were approved by the Ministry of Health & Welfare and Korean Stem Cell Research Center (institutional review board no. 78), and animal experiments were approved by the Institutional Animal Care and Use Committee of Korea University (KUIACUC-2010-143).

Additional Experimental Procedures

For more detailed and additional information on experimental procedures, please see Supplementary Materials and Methods.

Results

Hepatic Differentiation of Human ES Cells Using Lithium and Growth Factors

The Wnt/β-catenin signaling pathway is activated in the developing gut endoderm,[8] and activation of this signaling promotes the formation of mesendoderm from ES cells.[9,10] Lithium ion is known to inactivate GSK-3β and promote the stabilization and nuclear localization of β-catenin.[11] Immunohistochemical and Western blot analyses showed that lithium treatment of human ES cells differentiating as EBs (days 2–4) inhibits GSK-3β, activating β-catenin signaling (Supplementary Figure 1 *A* and *B*). Lithium exposure caused a moderate cell death, but no significant difference in the apoptotic rate was seen between lithium-treated and control EBs after an additional 2 days of EB formation in the absence of lithium (Supplementary Figure 1*C*). On the other hand, lithium treatment increased expression of genes known to promote mesendodermal and early hepatic fates (*Foxa2*, *Sox17*, *Mixl1*, *T*, *Prox1*, *Hex*, and *Hnf4*) (Supplementary Figure 1*D*). In contrast, the expression of neuroectodermal regulators (*Pax6* and *Sox1*) was reduced. Immunostaining of frozen EB sections showed that the proportion of cells coexpressing the neuroectodermal markers Pax6 and Nestin was reduced and the number of cells double positive for GATA4/Sox17 and for Foxa2/Sox17 was increased by lithium treatment (Supplementary Figure 1*E*).

When lithium-treated 6-day-old EBs were placed in collagen I–coated dishes in the presence of HGF, OSM, and DEX, many of the cells that migrated from EBs showed the polygonal morphology with distinct nuclei and expressed hepatic markers albumin and keratin 18 (Figure 1*A*). Reverse-transcription polymerase chain reaction analysis showed that all 3 factors were required to induce elevated expression of genes found in the fetal liver (Supplementary Figure 1*F*). Quantitative analysis showed that up to 69% ± 2% (n = 5) of the cells expressed both albumin and keratin 18 (Figure 1*B*), and some of the albumin-positive cells (8.1% ± 0.78%) expressed α-fetoprotein. Cells coexpressing biliary markers keratin 7 and 19 were also observed (15.3% ± 1.96% of total cells). The coexpression of albumin and keratin 18 in response to growth factor treatment was reproducibly seen with 3 human ES cell lines (Miz-hES-6, H9, and CHA-hES-3) and 2 human iPS cell lines (NIHi-7, NIHi-11; Figure 1*B*).

Many cells with polygonal morphology showed high γ-glutamyl transpeptidase activity and glycogen accumulation (Figure 1*C*). In addition, as the cells differentiated in the presence of growth factors (after day 6), urea secretion rapidly increased to match the levels achieved by HepG2 and primary human hepatocytes (Figure 1*D*; see Supplementary Figure 2 for further data on iPS cells).

Enrichment of Differentiated Hepatocyte-Like Cells

To identify human hepatocyte-like (HL) cells, we used indocyanine green (ICG), an organic dye that is uptaken and eliminated by hepatocytes, providing a nontoxic test used clinically to assess liver function. The spatial localization of cells labeled strongly with ICG suggested that laser microdissection and pressure capturing could be used to separate cells from regions with a high and low density of HL cells (referred to as ICG^{high} and ICG^{low}) (Supplementary Figure 3*A*). After laser microdissection and pressure capturing, the purified ICG^{high} cells showed an enhanced expression of hepatic markers (*ALB*, *AFP*, *AAT*, *TTR*, and *CPS1*) (Supplementary Figure 3*B*). Most of the cells in ICG^{high} clusters were positive for albumin/keratin 18, HNF4α/keratin 18, and albumin/cytochrome P450 1A2, and many cells were binucleate (Supplementary Figure 3*C*). Flow cytometric analysis revealed that 90% to 92% of the cells in purified ICG^{high} factions were positive for both albumin and HNF4α, whereas only 15.4% of ICG^{low} cells produced albumin (Supplementary Figure 3*D*). In addition, the albumin secretion rate of ICG^{high} cells (6.6 ± 1.5 μg/mL/24 h/10^6 cells) was comparable with HepG2 and human hepatocytes in vitro (Figure 1*E*). Quantitative polymerase chain reaction assessment showed that transcript levels of albumin and enzymes related to phases I and II of drug metabolism, CYP3A4 and GSTA1/2, were all enhanced in ICG^{high} cells compared with ICG^{low} cells (Figure 1*F*). Whereas undifferentiated ES cells have a few organelles, including electron translucent immature mitochondria, purified ICG^{high} cells formed bile canaliculi, together with