Research Article

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Evidence for multipotent endodermal stem/progenitor cell populations in human gallbladder

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Background & Aims: Multipotent stem/progenitor cells are found in peribiliary glands throughout human biliary trees and are able to generate mature cells of hepato-biliary and pancreatic endocrine lineages. The presence of endodermal stem/progenitors in human gallbladder was explored.

Methods: Gallbladders were obtained from organ donors and laparoscopic surgery for symptomatic cholelithiasis. Tissues or isolated cells were characterized by immunohistochemistry and flow cytometry. EpCAM+ (Epithelial Cell Adhesion Molecule) cells were immunoselected by magnetic microbeads, plated onto plastic in self-replication conditions and subsequently transferred to distinct serum-free, hormonally defined media tailored for differentiation to specific adult fates. *In vivo* studies were conducted in an experimental model of liver cirrhosis.

Results: The gallbladder does not have peribiliary glands, but it has stem/progenitors organized instead in mucosal crypts. Most of these can be isolated by immune-selection for EpCAM. Approximately 10% of EpCAM+ cells *in situ* and of immunoselected EpCAM+ cells co-expressed multiple pluripotency genes and various stem cell markers; other EpCAM+ cells qualified as progenitors. Single EpCAM+ cells demonstrated clonogenic expansion *ex vivo* with maintenance of stemness in self-replication conditions. Freshly isolated or cultured EpCAM+ cells could be differentiated to multiple, distinct adult fates: cords of albumin-secreting hepatocytes, branching ducts of secretin receptor+ cholangiocytes, or glucose-responsive, insulin/glucagon-secreting neoislets. EpCAM+ cells transplanted *in vivo* in immune-compromised hosts gave rise to human albumin-producing hepatocytes and to human Cytokeratin7+ cholangiocytes occurring in higher numbers when transplanted in cirrhotic mice.

Conclusions: Human gallbladders contain easily isolatable cells with phenotypic and biological properties of multipotent, endodermal stem cells.

Keywords: Stem cells; Liver regeneration; Gallbladder; Epithelial differentiation; Pancreatic islet cells.

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Abbreviations: hBTSCs, human biliary tree stem cells; PBG, peribiliary gland; hGSC, human gallbladder stem/progenitor cells; PAS, Periodic Acid Schiff; R-A, Rokitansky-Aschoff; EpCAM, epithelial cell adhesion molecule; PCNA, Proliferating Cell Nuclear Antigen; LGR5, leucine-rich repeat containing G protein-coupled receptor 5; PDX1, Pancreatic and duodenal homeobox 1; SOX, Sry-related HMG box; FC, flow cytometry; OCT4A, octamer-binding transcription factor 4 A; RT-PCR, real time polymerase chain reaction; cGMP, current good manufacturing practice; KM, Kubota's Medium; IF, immunofluorescence; K, keratin; NCAM, neural cell adhesion molecule; SR, secretin receptor; CD, cluster differentiation; HDM, hormonally defined medium; hHpSC, human hepatic stem cells; HDM-H, HDM for Hepatocyte differentiation; HDM-C, HDM for Cholangiocyte differentiation; HDM-P, HDM for Pancreatic islet cell differentiation; Mdr1, multidrug resistance protein 1; Mrp2, Multidrug resistance-associated protein 2; ICG, indocyanine green; TAT, tyrosine aminotransferase; CYP3A4, Cytochrome P450 3A4; SCID, Severely Combined Immunodeficient; HepPar-1, Hepatocyte Paraffin 1; FISH, Fluorescence *In Situ* Hybridization.

Introduction

Biliary tree stem/progenitor cells (hBTSCs) have been identified recently in peribiliary glands (PBGs) of normal extrahepatic bile ducts in humans and are able *in vitro* and *in vivo* to generate

mature cells of the hepato-biliary and pancreatic endocrine lineages [1–4]. Biliary tree tissue is available from fetal, neonatal, pediatric, and adult organs, including surgical materials, tissue routinely discarded from donor livers or pancreata. Gallbladder is an even more highly available tissue, since it is discarded from donor livers, and a huge number of patients are annually subjected to cholecystectomy. The aims of the present study were: (i) to characterize the phenotype and the location of human gallbladder stem/progenitor cells (hGSCs) and their niches, of endodermal origin, in fetal, neonatal, and adult gallbladder; (ii) to investigate the possibility of isolating and culturing hGSCs, (iii) to test their capability to differentiate towards multiple endodermal fates *in vitro*, and (iv) to assess their capability to repopulate liver parenchyma in a model of cirrhosis.

Materials and methods

Materials and methods used in this study are provided in the Supplementary Materials and methods.

Results

Analysis of human adult gallbladder tissue

In adult normal (N = 19) and pathological (N = 14) gallbladders, the surface epithelium was composed of a single layer of columnar cells. The mucosa is elevated into minute rugae, their projections into the lumen resembling intestinal villi. Mucosa folded in mucosal crypts, the so-called Rokitansky-Ashoff sinuses (R-A sinuses), that could be transversally cut and observed deeply in lamina propria (Supplementary Fig. 1). The mucosal crypts were deeper in pathological (668.5 ± 172.5 μm) than normal gallbladders (254.3 ± 78.1 μm; p <0.001). Accordingly, R-A sinuses were more extensive in pathological (16411.18 ± 4397.61 μm^2) *vs.* normal gallbladders (7626.6 ± 4191.4 μm^2; p <0.05). Gallbladders were devoid of PBGs, the stem cell niches of the biliary tree.

In normal gallbladders, the immunohistochemical analyses demonstrated EpCAM+ cells in the surface epithelium; EpCAM+ cells were located mostly within mucosal crypts with a gradient of expression from crypt (high) towards tip (low) of mucosal folds (Fig. 1A). In parallel, LGR5+ (Fig. 1A), SOX17+ (Fig. 1B), and proliferating PCNA+ (Supplementary Fig. 1) cells were also restricted into mucosal crypts. By double immunofluorescence (IF), EpCAM+ cells were mostly positive for LGR5 (67.72% ± 4.70, Fig. 1A), a marker of intestinal stem cells; a population of EpCAM−/LGR5+ cells was also present (7.97% ± 1.40). By confocal microscopy, EpCAM+ cells co-expressed nuclear transcription factors of bilio-pancreatic progenitors (PDX1, SOX17, Fig. 1B) and pluripotent stem cells (OCT4A, Fig. 1C). Interestingly, in mucosal crypts (R-A sinuses), immunohistochemistry on serial sections showed the presence of cells co-expressing pluripotency markers such as SOX2, OCT4A, and NANOG (Fig. 1C). The RT-PCR confirmed the expression of pluripotency genes and indicated that EpCAM+ cells expressed higher levels of pluripotency genes in comparison with cells negative for EpCAM (Fig. 1D).

When pathological gallbladders were examined (Supplementary Fig. 2), the number of EpCAM+ cells was higher in comparison with normal gallbladders (p <0.01); EpCAM+ (LGR5+, SOX17+, PDX1+) cells were not restricted to crypts but sprouted towards the apex of the mucosal rugae. On the other hand, similar to normal gallbladders, pluripotency genes (SOX2 and OCT4A) were still restricted to crypts (R-A sinuses). Accordingly, no differences in pluripotency gene expression were found by RT-PCR between normal and pathological gallbladders (Supplementary Fig. 2).

Isolation and culturing of stem/progenitors from adult gallbladders

After sorting for EpCAM, an average of 4–5 million cells were isolated from normal (4,988,333 ± 2,6; viability >90%) and pathological gallbladders (4,416,250 ± 2,4; viability >85%). The average number of viable cells isolated from normal *vs.* pathological gallbladders was not significantly different. The high standard deviation likely depended on the quality of tissues and the time elapsed between surgical removal and processing. The FC showed that, before sorting for EpCAM, 29.0 ± 12.4% of the freshly isolated cells were EpCAM+ (not shown). The immunomagnetic sorting enriched the EpCAM+ population to 69.34 ± 11.2% (p <0.05). In most of cases, we were able to obtain more than 90% of EpCAM+ cells (Fig. 2A).

Serum-free Kubota's Medium (KM), used with cells plated onto culture plastic, constituted self-replication conditions for these endodermal stem/progenitors and enabled enrichment of EpCAM+ cells to more than 90% (97.5 ± 3.9). The conditions are strongly selective for stem/progenitors and result quickly, within 5–7 days, in the disappearance of more mature cells, of both epithelial and mesenchymal origins [5].

FC on freshly isolated EpCAM-sorted cells, consistent with immunohistochemical findings, showed that EpCAM+ cells were mostly positive for LGR5 (69.5% ± 10.3) but, only a small sub-population (5.63 ± 2.54%) of EpCAM+ cells co-expressed several pluripotency markers (Fig. 2A), qualifying them to be probable stem cells; the other EpCAM+ cells, those with minimal expression of pluripotency genes are likely to be bipotent or multipotent progenitors including transit amplifying cells. The EpCAM+ cells from gallbladder, therefore, are a mix of probable stem cells, those with pluripotency genes, and multipotent progenitors, devoid of pluripotency genes, to be referred to as stem/progenitors.

At the moment of isolation, the contaminating cell populations, characterized by FC, consisted of cells of mesenchymal origin (white blood, hematopoietic, stromal, and endothelial cells), which constituted less than 5% of isolated cells (Supplementary Fig. 3).

The human gallbladder stem/progenitors (hGSCs/PCs) were also isolated according to cGMP protocols for potential therapeutic application.

Thereafter, a single cell suspension was obtained; cells were loaded with highly fluorescent Qdot® nanocrystals (Qtracker® 655 Cell Labeling Kit, Life Technologies Corporation) and plated at a clonal seeding density of 500 cells/cm^2 in a self-replication medium (KM) [6]. In these conditions, single individual hGSCs/PCs were capable of clonal proliferation; they started to proliferate after a 1–2 days lag period and formed small clusters of 10–15 cells after 6–8 days in culture (Fig. 2B). Colony formation efficiency, measured as the percentage of single seeded hGSCs/PCs that formed colonies, averaged 5% in KM and on culture plastic. After 14 days, large colonies were observed (Fig. 2C). Each colony was formed mostly by small (diameter = 8.84 ± 1.78 μm), densely packed, and uniform cells with high nucleus to cytoplasmic ratio