Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Multipotent stem/progenitor cells in the human foetal biliary tree

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Background & Aims: Biliary tree, liver, and pancreas share a common embryological origin. We previously demonstrated the presence of stem/progenitor cells of endodermal origin in the adult human extrahepatic biliary tree. This study evaluated the human foetal biliary trees as sources of stem/progenitor cells of multiple endodermal-derived mature fates.

Methods: Human foetal intrahepatic and extrahepatic biliary tree tissues and isolated cells were tested for cytoplasmic and surface markers of stem cells and committed progenitors, as well as endodermal transcription factors requisite for a liver versus pancreatic fate. *In vitro* and *in vivo* experiments were conducted to evaluate the potential mature fates of differentiation.

Results: Foetal biliary tree cells proliferated clonogenically for more than 1 month on plastic in a serum-free Kubota medium. After culture expansion, cells exhibited multipotency and could be restricted to certain lineages under defined microenvironments, including hepatocytes, cholangiocytes, and pancreatic islet cells. Transplantation of foetal biliary tree cells into the livers of immunodeficient mice resulted in effective engraftment and differentiation into mature hepatocytes and cholangiocytes.

Conclusions: Foetal biliary trees contain multipotent stem/progenitor cells comparable with those in adults. These cells can be easily expanded and induced *in vitro* to differentiate into liver and pancreatic mature fates, and engrafted and differentiated into mature cells when transplanted *in vivo*. These findings further characterise the development of these stem/progenitor cell populations from foetuses to adults, which are thought to contribute to liver and pancreas organogenesis throughout life.

Introduction

The biliary tree comprises intrahepatic and extrahepatic bile ducts [1] and shares a common embryological origin with the ventral pancreas. Liver, biliary tree, and pancreas share stem cell populations during the earlier stages of development when the anterior definitive endoderm forms the foregut [2–5]; these stem cell populations persist into adulthood and are located within the biliary tree [6–8]. The extrahepatic biliary tract originates directly from a portion of the ventral endoderm derived from a pancreato-biliary precursor expressing PDX-1 and SOX17 [5,9–13]. SOX17 expression determines the segregation of pancreato-biliary precursors [12].

Area and segmental ducts are large intrahepatic bile ducts [1,14] that share common histological features with extrahepatic bile ducts [14]. From an embryological perspective, there are several theories about the development of intrahepatic bile ducts, including their origination from in-growths of extrahepatic duct epithelium or development from hepatoblasts [11,15]. Interlobular bile duct formation during the early phase of embryologic development is derived from the differentiation of hepatic stem cells and hepatoblasts found in, or adjacent to, the ductal plate located around the forming portal tracts [2]. In adults, the canals

Keywords: Human foetal liver; Endodermal stem cells; Biliary tree; Liver; Pancreas; Biliary tree stem cells.

Received 13 February 2012; received in revised form 21 June 2012; accepted 5 July 2012; available online 20 July 2012

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Abbreviations: PDX-1, Pancreatic and duodenal homeobox 1; SOX17, SRY (sex determining region Y)-box 17; hHpSCs, human hepatic stem/progenitor cells; PBGs, peribiliary glands; hBTSCs, human biliary tree stem/progenitor cells; FBS, foetal bovine serum; KM, Kubota medium; DNAse, deoxyribonuclease; HDM, hormonally defined media; HDM-H, HDM for hepatocyte differentiation; HDM-C, HDM for cholangiocyte differentiation; HDM-P, HDM for pancreatic islet cell differentiation; SCID, severely combined immunodeficient; HepPar-1, hepatocyte paraffin 1; K, keratin; RT-PCR, real time polymerase chain reaction; EpCAM, epithelial cell adhesion molecule; AFP, α-fetoprotein; GGT, glutamyl transpeptidase; SR, secretin receptor; CD, cluster differentiation; PAS, periodic acid Schiff; cGMP, current good manufacturing practice; iPS, induced pluripotent stem cells; OM, original magnification; Ph-C, phase contrast.

of Hering, the remnants of the foetal ductal plate, are recognised as stem cell niches of the adult hepatic parenchyma, containing human hepatic stem cells (hHpSCs), hepatoblasts, and committed progenitor cells, referred to collectively as "hepatic stem/progenitors" [16–18]. We have recently identified stem cell niches of endodermal origin in the peribiliary glands (PBGs) of the human adult biliary tree [6–8]. Human biliary tree stem/progenitor cells (hBTSCs) are capable of giving rise to mature cells of the hepatic, biliary, and pancreatic islet lineages *in vitro* and *in vivo* [6].

However, the stem cell niches of the foetal biliary tree have not been characterised, or have their embryological connections to adult counterpart been defined [6–8].

Therefore, this study aimed to (i) identify the presence of endoderm-like stem/progenitor cells in extrahepatic and intrahepatic biliary trees (i.e., foetal hBTSCs) in foetuses; (ii) isolate hBTSCs from foetal extrahepatic and intrahepatic biliary trees; and (iii) demonstrate the pluripotency of foetal hBTSCs and their repopulation ability in the murine liver in experimental conditions.

Materials and methods

Reagents and growth factors were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. RPMI-1640 and foetal bovine serum (FBS) were obtained from Gibco/Invitrogen (Carlsbad, CA). Kubota medium (KM) is a wholly defined serum-free medium originally developed for culturing rodent hepatoblasts [19] that was subsequently demonstrated to be effective for human hepatic stem cells, hepatoblasts, and committed progenitors [20–22], and for biliary tree stem cells and progenitors from adult tissues [6].

Human foetal livers

The livers from human foetuses (16–22-week gestational age) were obtained by elective pregnancy termination (Supplementary Table 1) from the Department of Gynaecology (Sapienza, University of Rome, Italy). Informed consent was obtained from the mother before abortion. The study was approved by the local ethics committee of the Sapienza University Hospital.

Preparation of intrahepatic and extrahepatic biliary trees from foetal tissue

Intrahepatic and extrahepatic biliary trees were mechanically separated by dissection at the level of the left and right hepatic ducts. In this way, the extrahepatic biliary tree, which comprises left and right hepatic ducts, common hepatic duct, cystic duct, and gallbladder, was isolated.

The intrahepatic biliary tree, comprising ducts distal to the second order branches, was isolated as follows: the liver parenchyma was carefully mechanically detached with a scraper until a fine network of white ducts appeared. The finer branches of the biliary tree were successively purified from the remaining hepatocyte/hepatoblast aggregates with the scraper on the extreme periphery of the biliary tree.

Isolation of foetal biliary tree cell suspension

After mechanical dissection from the liver parenchyma, intrahepatic and extrahepatic biliary tree specimens were enzymatically digested in buffer containing 300 U/ml type I collagenase and 0.3 mg/ml deoxyribonuclease (DNAse) for 20–30 min at 37 °C. This procedure produced a homogeneous suspension of cell aggregates passed through an 80-μm mesh filter. Viable cell counts were determined by the trypan blue dye exclusion test.

Isolation of foetal liver parenchymal cell suspensions

Foetal liver parenchymal cell suspensions were isolated as described by Reid *et al.* [23]. After mechanical dissection of the biliary tree tissue, the remaining liver parenchymal tissue was partially digested with enzymes (300 U/ml type I collagenase and 0.3 mg/ml DNAse); this allowed parenchymal cells to remain in clumps that were centrifuged by very low-speed spins, leaving the supernatant containing the free-floating haemopoietic cells. After debulking the tissue from haemopoietic cells, clumps of parenchymal cells were subjected to further enzymatic digestion to yield either small aggregates or single-cell suspensions optimal for the selection of hepatic stem cell or hepatoblast subpopulations. Viable cell counts were determined using the trypan blue dye exclusion test.

Cell cultures

Approximately 3×10^5 cells obtained from either biliary tree or liver parenchymal tissue were seeded onto 3-cm plastic culture dishes and maintained overnight (approximately 12 h) in KM with 10% FBS. Thereafter, cell cultures were maintained in serum-free KM and monitored for at least 2 months. KM is a hormonally defined medium (HDM) optimised for *ex vivo* expansion of endodermal stem/progenitors, as stated earlier [6,19]. KM allows proliferation of early lineage stages of mesenchymal cells (e.g., angioblasts, endothelial cell precursors, and hepatic and pancreatic stellate cell precursors) under serum-free conditions if supplemented with 10 ng/ml of leukaemia inhibitory factor, vascular endothelial cell factor, epidermal growth factor, and stem cell factor each; however, even with these supplements, does not allow the growth mature mesenchymal cells [22].

KM and HDMs for differentiation [6] (i.e., HDM-H for hepatocytes, HDM-C for cholangiocytes, and HDM-P for pancreatic islet cell differentiation) [22,24] were prepared as described in Supplementary data.

Measurement of C-peptide secretion under low and high glucose concentrations

The cultures were subjected to functional tests to determine C-peptide secretion in response to glucose, as described previously [6]. The procedure is described in detail in Supplementary data.

Transplantation of biliary tree cells into the livers of severely combined immunodeficient (SCID) mice

hBTSCs isolated from the intrahepatic or extrahepatic biliary tree were transplanted via intrahepatic injection in the quiescent livers of 6- to 7-week-old SCID mice (n = 4). Their engraftment and differentiation were evaluated by morphology and immunohistochemistry, as described previously [6]. hBTSCs from intrahepatic or extrahepatic foetal biliary trees (5×10^5 cells) were injected into the frontal lobe of the liver. Four mice transplanted with KM only were used as controls. To exclude any tumour formation within the liver or other organs, 4 mice were followed-up for 3 months after transplantation [25]. All the animals received human care, were closely monitored until recovery, and were allowed free access to food and water. All the animal protocols complied with our institutional guidelines. No mortality occurred. Mice were killed 1 month after transplantation, their livers were excised, and tissue fragments were fixed for immunohistochemical analysis.

Anti-human antibodies that do not react with mouse antigens were used to test engraftment and differentiation of foetal hBTSC in mouse livers. In particular, anti-human HepPar-1 and anti-human albumin were used to investigate the presence of human hepatocytes derived from transplanted cells [6]; anti-human mitochondria [26], keratin (K) 7 [6], and K19 were used to investigate the incorporation of human-derived cholangiocytes within mouse bile ducts. The human hepatocyte mass represents the percentage of parenchymal area occupied by human hepatocytes; it was measured by an image analysis system (IAS-Delta Sistemi, Rome, Italy) and calculated as the area occupied by anti-human HepPar1-positive hepatocytes–total parenchymal area × 100 [6]. The number of human cholangiocytes was calculated as the percentage of anti-human mitochondria and/or K7-positive cholangiocytes within the bile duct with respect to the total number of cells lining the bile ducts. All counts were performed in 6 non-overlapping fields (20× magnification) for each slide; at least 3 different slides were taken from each liver fragment [6].

The complete methods for light microscopy, immunohistochemistry, immunofluorescence, reverse-transcription polymerase chain reaction (RT-PCR), and statistical analysis are included in Supplementary data.

Results

Phenotype of cells in the foetal biliary tree

Extrahepatic biliary tree specimens comprised common hepatic ducts, cystic ducts, and gallbladders. Small evaginations of