From hepatocytes to stem and progenitor cells for liver regenerative medicine: advances and clinical perspectives

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Abstract

The parenchymal liver cell is a unique fully functional metabolic unit that can be used for liver regenerative medicine to restore function of the diseased organ; the aim of the procedure is to prevent progression of end-stage disease. The alternative, orthotopic liver transplantation, is highly intrusive, irreversible and limited by general organ shortage. Mature liver cell – hepatocyte – transplantation has been shown to have short- to medium-term efficacy for correction of miscellaneous inborn errors of metabolism. However, although proof of concept has been established, the procedure has not yet achieved full success, due to limited durability of functional benefit. Hepatocyte procurement is also restricted by organ shortage, and their storage is difficult due to poor tolerance of cryopreservation. Alternative cell sources are therefore needed for development and wider accessibility of cell-based liver regenerative medicine. Besides safety, the main challenge for these alternative cells is to acquire similar levels of functionality once implanted into the target organ. In this respect, liver derived progenitor cells may have some advantages over stem cells derived from other tissues.

Current status of liver cell transplantation

The concept of liver cell transplantation (LCT) entered clinical practice slightly more than 10 years ago. The technique aims to treat not only a variety of inborn errors of liver metabolism but also fulminant liver failure, using mature hepatocyte intraportal infusions (1–4). Proof of the concept has been established with replacement of missing

function by infusing liver cell suspension into diseased liver, and most favourable results have been obtained with miscellaneous inborn errors of metabolism, such as Crigler Najjar syndrome (1,5), urea cycle defects (6–8), glycogen storage disease (9), clotting factor deficiencies (10) and Refsum disease (2). Such clinical reports have demonstrated that LCT can restore metabolic liver function for up to 18 months post-infusion or more. Repeated doses of hepatocyte infusions in a child with urea cycle disorder and important secondary psychomotor retardation has led to demonstration of donor cells in liver biopsies up to 8 months after the last infusion, restoring *de novo* activity of arginosuccinate lyase (7). In these studies, infused cells contributed to restoration of liver metabolism as well as improvement of psychomotor development (7).

Key advantages of LCT in comparison to orthotopic liver transplantation (OLT) are that the method is less invasive, is fully reversible, and minimizes risks related not only to surgery, but also to lack of function of the graft, or long-term graft loss. In addition, the method does not prevent possibility to perform OLT later; this has been carried out without complication in children having undergone previous LCT. As LCT is based on allogeneic cells, patients need to receive immunosuppressive treatment following infusions.

To prevent thrombosis at the site of an infusion, hepatocytes are infused slowly, with addition of heparin and *N*-acetylcystein, shown to inhibit tissue factor-dependent procoagulant activity of hepatocytes (11). A further safety issue is possible bacterial contamination of fresh hepatocyte preparations, unknown at the time of infusion, as is also observed for whole organs; this may be addressed by use of prophylactic antibiotherapy covering Gram-negative bacterial strains during the infusion procedure. All these clinical studies have revealed both feasibility and safety of LCT, with absence of serious adverse events reported.

One main limitation for use of mature liver cells is that mature human hepatocytes do not tolerate cryopreservation well. Cryopreservation of hepatic cells induces severe

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impairment of cell adhesion, morphological changes in mitochondria, loss of ATP production, alteration in mitochondrial respiratory chain enzymes, increased mitochondrial permeability and loss of membrane potential. Cytochrome C is released into hepatocyte cytoplasm, and this may lead to cell death by apoptosis (12). New perspectives using vitrification coupled to, or not coupled to, encapsulation are now under investigation. Although some success has been reported with cryopreserved cells (6,13), clinical experience has shown them to be less efficient unless perhaps by selecting high quality cells from young donors (13), which is rarely achievable in routine transplantation.

The storage limitations, added to the organ shortage issue, lead us to consider other cell sources such as stem cells as an alternative for liver cell therapy. Stem/progenitors cells are undifferentiated cells that demonstrate high ability of self-renewal and potential of expansion *in vitro*. Adult tissue-derived stem cells are safer than embryonic, foetal or induced pluripotent cells and seem closer to clinical applications; also, no ethical issues are raised by their use. Also, stem cells have a good reputation for resistance to cryopreservation. The prospect is that these cells can soon be used for cell therapy of the liver, after induced or spontaneous differentiation into functional hepatocytes (4,14,15).

Extrahepatic sources of stem cells

Bone marrow and haematopoietic tissues

Adult bone marrow contains different cell populations including mesenchymal stromal cells, endothelial cells, fibroblasts and haematopoietic cells. In different studies, it has been postulated that these cells would be able to improve hepatic function when suitably prepared. Transplantation of whole bone marrow mononuclear cells or selected haematopoietic populations, into irradiated or liver injury-induced mice, has demonstrated the presence of donor cells in recipient liver displaying hepatocyte-like morphology (16–19).

Most interestingly, in a mouse model of tyrosinaemia, infusion of bone marrow from wild-type mice was able to restore fumarylacetoacetase activity in hepatocytes from *fah*^{−/−} mice, as a consequence of fusion mechanisms between wild-type bone marrow cells and *fah*^{−/−} recipient hepatocytes (17,19). However, fusion between bone marrow cells and hepatocytes in other studies has not been found, and in particular, fusion mechanisms have not so far been reported in humans (4,20,21).

Observed improvement in liver metabolic function after bone marrow or haematopoietic cell transplantation following liver injury, could also be the result of activity

of cytokines or secreted growth factors (secretosomes), that modify the local microenvironment and contribute to hepatocyte proliferation/function, more than to *de novo* hepatocyte function of transplanted cells. This mechanism, suggested in other tissue repair mechanisms, has been suggested in a mouse model of toxic induced fulminant hepatitis (22), but cannot explain *de novo*-acquired metabolic function in models of inborn errors of liver metabolism. Peripheral blood monocytes are a further cell source that has been used to obtain adult-derived hepatocyte-like cells, and these are currently being tested for their drug-metabolizing capacity and for use in regenerative medicine (23,24).

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) were first described by Freidenstein *et al.* as plastic-adherent fibroblast-type cells with high proliferative and capacities of differentiation, into the osteogenic lineage (25). MSCs have since then been isolated from various tissues such as the skin, Wharton's jelly, adipose tissues, amniotic membrane of the placenta and foetal tissues (26,27,28). From a clinical perspective, uses of foetal tissues or embryonic tissues as potential sources of stem cells are limited by ethical considerations, and possibly by higher risk of uncontrolled proliferation and teratoma formation. For these reasons, adult-derived stem cells are currently preferred for clinical use.

In vitro studies have demonstrated the ability of MSCs of different origins to differentiate into hepatocyte-like cells when specific growth/differentiation factors are added to their culture medium (29,30). After several days, cells display morphology and expression profiles identical to native immature hepatocytes. Undifferentiated MSCs isolated from Wharton's jelly demonstrate an intermediate phenotype profile between hepatoblasts and mature hepatocytes (31,32). Such undifferentiated cells constitutively express albumin, α -foetoprotein and connexin-32 as well as cytokeratins -8, -18 and -19. In the same study, partially hepatectomized mice transplanted with undifferentiated MSCs demonstrated their engraftment and maintenance over 6 weeks post-injection, with persistence of typical hepatic markers.

Another suitable source of MSCs is adipose tissue. Human adipose tissues contain a relatively high number of MSC (hADSC) which can be derived easily from plastic surgical bi-product material (plastic surgery waste material) (33). These share similar phenotype (CD13⁺, CD29⁺, CD90⁺, CD105⁺, CD44n⁺, CD49e⁺, CD54⁺, CD71⁺, CD117⁺, CD166⁺, SH2⁺, SH3⁺, STRO-1⁺) and immunological [MHC I^{low}, MHC II[−] (HLA II)[−]] features with bone marrow-derived MSCs, making them an attrac-

tive source for allogeneic transplantation. Recent research suggests the presence of a hepatic cell subpopulation residing within heterogeneous adipose-derived progenitor cell pools (34). Hepatic differentiation potential of these has lately been confirmed with acquisition of functional activities such as albumin production, glycogen storage, CYP expression, low-density lipoprotein uptake, urea cycle and drug metabolizing activities. Subsequent transplantation into nude mice with acute liver injury has resulted in restored liver function, while markers of liver injury were lowered (35). hADSC have been shown to have higher engraftment capacity in immunocompromised mouse livers when pre-differentiated into hepatocytes before infusion, displaying human proteins such as albumin and HepPar. Engrafted cells were mainly hepatocyte-like, having negative expression of the biliary marker CK19. Cell proliferation level was low (<1%) while engrafted cells established connections between themselves and with surrounding mouse hepatocytes (36).

Besides their high potential of differentiation, MSCs have been demonstrated to have immunosuppressive properties, and also being able to reduce inflammation (37). Recent investigations exploring infusion of co-encapsulated hepatic and mesenchymal cells have demonstrated improvement in liver function after transplantation (38).

Intrahepatic sources

For hepatologists and transplant clinicians, it seems logical to seek a candidate liver progenitor that would already be resident in the adult liver. This approach may be restricted by lack of human liver tissue available, once more, because of organ donor shortage. Mature human hepatocytes are themselves involved in liver tissue regeneration, having the capacity to proliferate and repopulate liver after acute injury (39). Besides this mechanism, different populations of stem/progenitor cells participate in replacement of liver cells lost including, oval cells, small hepatocytes, liver epithelial cells and mesenchymal-like cells. Presence of cytokines produced by non-parenchymal cells are also needed for hepatocyte proliferation itself.

In the rodent, oval cells are progenitor cells located in the walls of bile ductules and canals of Hering, which are able to generate both hepatocytes and bile duct epithelial cells. They constitute the progenitor niche or reservoir of the liver. In response to injury, these cells proliferate and migrate into the liver parenchyma. This regenerative step is evidenced by an important increase in α FP expression and important extracellular matrix remodelling (39,40). Hepatic stellate cells are a further cell type that may play a role and favour differentiation of oval cells into mature hepatocytes (41). Morphologically, oval cells are small in size with high nucleus/cytoplasmic ratio. Oval cells

express not only mixed hepatocyte/biliary markers but also haematopoietic-associated antigens such as CD34, c-Kit (CD117) and Thy-1. Although oval cells are attractive candidates for hepatocyte differentiation, they are not currently isolated nor deliverable as cell suspensions, and are not therefore used in human liver regenerative medicine.

Liver epithelial cells (LECs) may be derived from primary culture of human adult hepatic tissue and appear several days after death of hepatocytes. *In vitro*, they can be expanded and demonstrated to have potential of differentiation into both liver cell types – hepatocytes and biliary cells – with respective specific antigen expression. Phenotypic analysis has also demonstrated their common expression of mesenchymal and haematopoietic markers. However, it is not possible to detect the cells by their histology in the adult liver due to their absence of specific markers. Finally, LEC differ from oval cells by absence of expression of CD34 antigen and by their polygonal shape morphology (42). Small hepatocytes are isolated from the non-parenchymal cell fraction and display high proliferative potential, but with limited *in vitro* differentiation into mature hepatocytes. These cells appear after long periods in culture and their origin is not fully understood (43).

Presence of mesenchymal-like cells also called adult human hepatocyte stem/progenitor cells (ADHLSCs) has also been identified in hepatocyte suspensions obtained after collagenase perfusion of normal adult liver (44). These cells are isolated from primary culture of hepatocytes and demonstrate an important potential of proliferation and a more advanced and complete morphological and functional differentiation into hepatocytes. The cells do not express haematopoietic markers but express mesenchymal marker proteins such as vimentin and alpha smooth muscle actin, as well as albumin, alpha foetoprotein and alpha 1 antitrypsin. In contrast to other MSCs, their differentiation into osteogenic and adipogenic cells is lost, suggesting that these cells are already engaged in the hepatocytic lineage, being more like progenitors than like stem cells. Khuu *et al.* demonstrated advanced liver metabolic activity of differentiated ADHLSC. *In vitro* differentiated cells were able to metabolize glucose in the presence of lactate and pyruvate, associated with increased expression of specific enzymes including G6P, PC and PECK. More interestingly, these differentiated ADHLSC were found to be able to metabolize ammonium, conjugate bilirubin and express phase I and phase II enzymes responsible for metabolism of both exogenous (that is, as a drug) and endogenous (bilirubin) compounds express CK19, an oval cell marker also present on cholangiocytes or even on hepatocarcinoma cells. Transplantation of these undifferentiated ADHLSCs into rodents was followed by *in vivo* differentiation and

synthesis of human albumin 6 weeks later, while the cells showed long-term engraftment potential (45).

In addition, ADHLSCs lack expression of HLA-Dr and are tolerant of cryostorage. HLA-Dr is a cell surface molecule that modulates graft rejection. Recent clinical trials using MSC, HLA-Dr-negative cells, have demonstrated their immunosuppressive effect in graft-versus-host disease and their clinical safety (37). Taken together, differentiated or not, ADHLSC could represent an excellent candidate model for liver cell therapy. Safety pre-clinical experiments also demonstrate the absence of tumorigenicity of these cells *in vitro* or *in vivo* (Scheers I, Maerckx C, Najimi M, Sokal E submitted) in comparison to HepG2 cells, a human liver carcinoma cell line. ADHLSCs are anchorage dependent and do not grow on soft agar; they have limited proliferation capacity and do not express oncogenes. In the same way, the safe profile of ADHLSC cells has been demonstrated by absence of tumour formation *in vivo*, after injection in nude mice (Scheers *et al.* submitted). Functionality assays have shown that the cells display all major phase I and phase II drug metabolizing enzymes, confirming further their comprehensive hepatocyte-like properties and leading to consideration also that ADHLSC could be used in *in vitro* metabolic and toxicity studies of new chemical compounds (45). Among other safety issues, distribution of ADHLSC after their injection into animals has to be investigated. Studies of such biodistribution can be performed using small-animal PET (positron emission tomography) imaging. Viral vectors such as adeno- or retroviral- can be used to transfect rat MSC – with thymidine kinase reporter genes that then are easily detected after phosphorylation of guanidine compounds (^{18}F -FHBG). This method has not demonstrated toxic effects on MSCs regarding viability, proliferation and differentiation potentials (46). In the clinic, hepatocyte distribution in liver has been shown by labelling with indium 111, and this technique may be easily applied to stem or progenitor cell transplantation I, the coming clinical trial (47).

Conclusion

In the human population, there is medical need to restore liver function in case of its sudden loss during acquired diseases, or in cases of a single missing function such as in inborn metabolic diseases. Liver cell-based therapy and stem cell technology may soon address this issue. Mature hepatocyte transplantation has already been shown to be efficient in restoring missing function, but so far efficacy is only partial and suitable alternative sources of cells is needed to replace hepatocytes. This would also alleviate current problems of organ shortage and establish the techniques more easily, to be available in more liver transplant centres.

One particular source of cells has demonstrated promising results: adult liver-derived progenitor cells, as they have a safe profile and have already been shown to be engaged into the hepatocyte lineage. They have a more pronounced potential for differentiation into mature hepatocytes, expressing all desired functions to repair diseased liver. In addition, besides their clinical perspective in liver regenerative medicine, the extensive pharmacological properties of the cells render the model an excellent candidate to be used *in vitro* for ADME toxicology studies in the pharmaceutical industry.

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