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Transplantation of primary and reversibly immortalized human liver cells and other gene therapies in acute liver failure and decompensated chronic liver disease

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Studies performed in experimental small animals with hepatic-based metabolic disorders but no structural liver disease, including Gunn and analbuminaemic rats and rabbits with inherited low-density lipoprotein receptor deficiency, have shown that up to 95% of hepatocytes transplanted into the spleen or liver remain in these sites, with improvement in metabolic function of recipients[1-4]. The feasibility of hepatocyte transplantation as a clinically-relevant therapeutic tool has subsequently been demonstrated in a small number of patients with disorders such as Crigler-Najjar syndrome type 1[5], ornithine transcarbamoylase deficiency[6] and familial hypercholesterolaemia[7], in whom the delivery of numbers of primary hepatocytes approximately equivalent to only 5% or less of the normal liver cell mass led to satisfactory, if incomplete, correction of the metabolic defect. Hepatocyte transplantation has several real and potential adv antages over conventional orthotopic liver transplantation (OLT). In addition to the relative simplicity, less invasive nature and lower associated cost of the former intervention, the ability to cryopreserve primary liver cells without substantial loss of viability or physiological function on subsequent thawing, provided that they are attached to microcarriers or gel-entrapped as spheroids[8-10], offers the potential that, through liver cells stored in centralised banks, it will be possible one day to have them widely available for clinical use within a few hours of a decision to institute such therapy.

A particularly important issue is whether liver cell transplantation has a role in the management of patients with severe liver damage resulting in acute liver failure (ALF), where only short-term support may be required given the potential for regeneration of the native liver[11], or those with decompensated chronic liver disease, in whom on-going support over a longer period may be necessary. The availability of effective liver support "on demand" would constitute an important advance in the management of such patients, given the rapidity with which irreversible clinical deterioration often occurs in these settings. In this review we consider clinical experiences with hepatocyte transp lantation in each of these liver failure syndromes reported to date, as well as discussing issues pertaining to the ideal cell type for clinical use. The potential applications of adjuvant and other gene therapies, as recently described experimentally, which may overcome two of the major limitations of liver cell transplantation at present, namely the relative unavailability of primary human hepat ocytes and the requirement for pharmacological immunosup-pression in the post-transplantation period, are also considered.

Liver cell transplantation in experimental animals with ALF due to chemically-induced hepatic necrosis or surgical models of hepatic ischaemia or resection has, to date, generally involved the use of primary hepatocytes. Such treatment has been associated with improved survival, even when small numbers of cells, in the order of only 0.5% to 3% of the normal hepatocyte mass, are used[12-19]. These small numbers of cells may be sufficient to enhance hepatic regener ation, on which recovery ultimately depends, possibly due at least in part to reduction in levels of transforming growth factor (TGF)β1, a potent inhibitor of this process[20]. However, cell transplantation has been performed prior to the induction of ALF in some surgical models, a scenario clearly incongruous to the clinical setting. Furthermore, since mediators such as interleukin-6 and tumor necrosis factor-α produced by activated Kupffer cells in ALF contribute to both progressive liver damage and the development of multi-organ failure[21-23], while the liver is also the source of TGF-β1 and other

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inhibitors of liver regeneration[24,25], the relevance of animal models in which the liver is removed to the clinical situation, in which it remains *in situ*, is uncertain.

Nevertheless, several preliminary studies have already been carried out on the clinical efficacy of hepatocyte transplantation in adult and paediatric patients with ALF. No randomised, controlled data is yet available and the impact of such treatment on clinical course is uncertain. Strom *et al*[26] reported their experience at the Medical College of Virginia with transplantation of small numbers (0.1% or less of the normal hepatocyte mass) of ABO-matched, freshly isolated or cryopreserved primary human hepatocytes via injection into the splenic artery in two adults with ALF due to hepatitis B virus infection and phenytoin hepatotoxicity, respectively. Both patients were in grade IV encephalopathy prior to the treatment. Immunosuppression was with methylprednisolone and cyclosporin. Reduced blood ammonia levels along with improvement in encephalopathy grade and reversal of haemodynamic instability were noted. Other evidence of metabolic function was unimpressive, with the serum bilirubin level increasing in both patients and the prothrombin time rising in one and falling only marginally in the other. Both patients underwent OLT after three and 10 days, respectively, with subsequent full recovery. None of three control patients, for whom either fresh or frozen hepatocytes were not available or consent for hepatocyte transplantation could not be obtained, survived. Of an additional five adult patients with ALF who unde rwent hepatocyte transplantation in this way at the same centre, three (60%) in grade III to IV encephalopathy (due to acetaminophen toxicity: $n = 1$; hepatitis B virus infection: $n = 1$ and idiopathic: $n = 1$) were successfully "bridged" to OLT between one and five days later. Another patient in grade IV coma (due to herpes simplex virus infection and/ or sodium valproate hepatotoxicity) died of sepsis-related cardiovascular instability on day 5 before OLT could be performed, while the remaining patient with acute hepatitis B virus infection recovered without OLT but had only low-grade in grade I encephalopathy prior to the procedure[27]. Bilir *et al*[28] from the University of Colorado subsequently transplanted by percutaneous injection larger numbers of cryopreserved primary human hepatocytes (in the order of 5% of the normal hepatocyte mass) in five adult patients with ALF and grade IV encephalopathy who were not candidates for OLT. Three (60%) patients survived more than 72 hours, with evidence of improved encephalopathy score, serum ammonia levels and prothrombin times. A delay in the order of 24 to 72 hours was apparent between hepatocyte transplantation and the first biochemical or clinical sign of improvement, possibly reflecting the time required for effective engraftment, as demonstrated in experimental animals[29]. However, no patient survived for more than seven weeks. The same group reported technical success in achieving engraftment via the transjugular route in an additional comatose patient with ALF, although no clinical benefit could be demonstrated[30].

Transplantation of comparably small numbers of hepatocytes has been performed in six children with ALF, aged six months to 15 years, with aetiologies including acetaminophen hepatotoxicity ($n = 1$), other drug reactions ($n = 2$), idiopathic ($n = 2$) and sepsis/total parenteral nutrition ($n = 1$)[26,27,31]. Five (83%) of these children were in grade IV encephalopathy at the time of treatment. Of these, four (80%) died within seven days, despite instances of reduction in serum ammonia levels and requirement for coagulation factor support, while the other patient recovered without OLT, having received multiple infusions of hepatocytes over a three day period. Another child, in whom hepatocyte trans plantation was performed when in grade I encephalopathy, underwent OLT two days later.

In experimental animal models, successful hepatic engraftment of transplanted hepatocytes is associated with evidence of transient micro-circulatory damage to the host liver[32]. This development of reversible portal hypertensi on precludes the transplantation of larger quantities of hepatocytes, at least in one session[33]. However, transplantation of a relatively small number of cells can lead to substantial replacement of the recipient's liver mass in the experimental setting in the presence of various regenerative stimuli. The latter include the concurrent intravenous injection of hepatocyte growth factor (HGF)[34], induction of ischemic atrophy of the contralateral liver lobe[3,4] and in situations in which the recipient's own liver cells have a shortened life-span, as occurs with acute chemical injury[35], transduction with a recombinant adenovirus vector expressing a non-secreted urokinase[36] or anti-Fas antibody-induced apoptosis[37].

The growth advantage of transplanted cells over native cells demonstrated in these circumstances may be maximised in the ALF setting by the use of purified hepatic stem cells able to replicate at least 100 times without loss of function or malignant transformation, as identified in adult rodent liver[38,39]. Repopulation experiments using purified fractions of total liver cell suspensions will be required to identify any such cells in the adult human liver[40]. Of note, a bone marrow-derived stem cell capable of repopulating the liver with mature hepatocytes following hepatic injury has recently been described in rodents. In the order of 1.0×10^6 hepatocytes (approximately 0.1% of the total hepatocyte mass) originated from transplanted

bone marrow cells by day 13 after liver injury[41]. This finding raises the exciting possibility that bone marrow infusion may have therapeutic potential in liver failure, although, even if extrapolated from the animal to the human situation, the time required for engraftment and cell differentiation would be problematic in the acute setting.

On the premise that the fetal liver contains epithelial cells that are in different stages of lineage progression, some of which may exhibit the full regenerative potential of stem cells, transplantation of fetal hepatocytes has been suggested as the way forward, rather than use of adult cells. Clinical data so far are limited. Habibullah *et al*[42] in India transplanted 6×10^7 blood group-matched, fetal hepatocytes per kilogram body weight by intraperitoneal injection in seven patients with ALF of unspecified aetiology and grade III to IV encephalopathy. Three (43%) transplanted patients survived. Those who survived each had a prothrombin index of 1.5 or less and were in grade III or IV a encephalopathy at the time of hepatocyte transplantation, while those who died had a more severe illness marked by prothrombin indices ranging from 2.6 to 3.0 and grade IVb encephalopathy at this time. Blood ammonia concentrations fell in 5/6 (83%) transplanted patients in whom serial levels were obtained. Survival in a control group, selected on the basis of inability to procure consent for the hepatocyte transplantation procedure, was 33%. Whether fetus-derived hepatocytes will prove superior to their adult counterparts in terms of regenerative capacity and functional characteristics, and whether any such superiority will translate into increased clinical efficacy in the ALF situation or with chronic liver damage, remain to be determined.

Irrespective of the fetal or adult nature of transplanted hepatocytes or even the use of bone marrow infusion as a source of stem cells capable of repopulating the liver, the potential role in the ALF setting of co-transplantation of non-parenchymal cells requires consideration. The co-transplantation of such cells may be advantageous to the viability and function of transplanted hepatocy tes in terms of secretion of extracellular matrix, analogous to the beneficial effects in these regards documented in *ex-vivo* culture systems[43-45]. However, the inclusion of non-parenchymal cells in suspensions for transplantation might also have deleterious effects if these cells become activated to produce cytokines such as TGF-β1 and interleukin-1 which promote apoptosis of hepatocytes and inhibition of liver regeneration[24,25].

Controlled studies in which liver cell transplantation in its various forms is compared to standard intensive care will be required in order to determine the ef ficacy or otherwise of this intervention in the ALF setting. These studies should be performed, at least in the first instance, in the most severely affected groups fulfilling criteria for OLT but for whom this is unavailable or contraindic ated by co-morbidity. Such studies will need to be conducted on a multicentre basis and using standardised outcome measures if sufficient numbers of patients are to be recruited and meaningful results obtained. If benefit is demonstrated in such groups, efficacy should then be addressed in patients with apparently lesser degrees of liver damage not fulfilling OLT criteria, as recent analyses showing poor negative predictive values of current selection criteria for OLT indicate that a substantial number of such patients nevertheless deteriorate with intensive medical care alone[46-48].

Clinical experience to date with liver cell transplantation for decompensated chronic liver disease is similarly limited, uncontrolled and confined to the use of primary adult hepatocytes. The feasibility and apparent safety of hepatocyte transplantation in chronic liver disease was first reported by Mito and Kusano in Japan[49], who injected small numbers of freshly isolated human cells into the spleens of 10 patients with cirrhosis or chronic viral hepatitis at laparotomy and under the influence of epidermal growth factor. Viable hepatocytes were detected in the spleen by scintigraphy in almost all patients up to 11 months later. Four patients with cirrhosis subsequently underwent hepatocyte transplantation via injection into the splenic artery at the Medical College of Virginia[27]. One of two patients in grade IV encephalopathy survived until OLT was performed on day 2, while a third patient in grade II-III encephalopathy recovered to be discharged from hospital before relapsing and dying on day 33. Another patient in whom liver failure with grade IV encephalopathy was precipitated by triseg-mentectomy died of cardiovascular instability within 2 days of the procedure. More rece ntly, Bilir *et al*[50] in Colorado treated five patients with Child's C cirrhosis who were not candidates for OLT with transplantation of 1×10^9 to 1×10^{10} cryopreserved primary human hepatocytes (in the order of 5% normal liver cell mass), with viability of 52% to 73% after thawing, via infusion into the splenic artery. Immunosuppression was with cyclosporin. Indications for hepatocyte transplantation were refractory encephalopathy with ascites (n=4) and hepatorenal syndrome (n=1). Improvements in encephalop athy grade and ascites and renal function, respe ctively were noted within days of the procedure. Improvements in serum albumin levels and prothrombin times also occurred, but only after a period of four to six months. Aside from one patient who stopped immunosuppression after five months, all patients were alive and well two to 15 months post-transplantation. There were no complications related to the procedure. In particular, no overt evidence of increased portal

venous pressure, as reported following hepatocyte transpla ntation in experimental animals with cirrhosis[51], was recorded, although it is to be noted that transjugular intrahepatic portosystemic shunts had been placed prior to transplantation in the majority of patients. No instances of pulmonary vascular complications were clinically apparent, despite concerns of increased pulmonary sequestration of transplanted cells in the context of portal-systemic shunting. Results of a randomised trial of hepatocyte transplantation in cirrhosis, which is currently in progress, are awaited.

Even if the efficacy of primary human hepatocyte transplantation was to become firmly established in the ALF and decompensated chronic liver disease settings, the limited availability of these cells is likely to represent a severe, ongoing impediment to its widespread clinical application. Primary human hepatocytes are obtained from resected surgical specimens, unused segments or end-lobe wedges of donor organs for OLT and livers from apparently healthy donors which are ultimately rejected for OLT on account of overt steatosis, since these are often also fibrotic. Although approximately 10^9 hepatocytes are readily obtained from a segment of normal liver, the yield is substantially reduced when fibrosis is present[52]. The high demand for whole organ OLT and the expertise to transplant portions of a single organ into more than one recipient mean that completely normal liver is increasingly in short supply for other uses. A satisfactory number of viable primary human hepatocytes cannot at present be obtained from needle biopsy-sized fragments of liver.

The *ex-vivo* expansion of hepatocytes recovered from surgical specimens represents a potential means of overcoming the problem of limited hepatocyte availability for transplantation. A number of proliferating human cell lines which have become immortalised by virtue of cultural conditions, without the use of oncogenes or carcinogens, have now been maintained in continuous culture for up to several years, although considerable limitations in their spectra of metabolic activity may inhibit their clinical utility were they to be used in the liver failure setting[53-56]. An alternative approach to achieving population expansion of hepatocytes *ex-vivo*, without necessarily compromising their differentiated functional capability and the prospect of obtaining meaningful clinical support following their subsequent transplantation, is to transfect the cells with a replication-deficient retrovirus carrying a temperature-sensitive variant of the simian virus 40 large tumor (SV40 large-T) antigen gene. This gene binds to the cell cycle control protein, p53, and produces cell lines which proliferate at 33℃ but cease proliferating and develop enhanced differentiated function, including upregulated synthesis of α-1-antitrypsin and inducible activity of some cytochrome P450 isoforms, at 39℃[57,58].

Hepatocytes from Gunn rats immortalised by transfection with the SV40 large-T antigen have been used successfully for *ex-vivo* gene therapy, in which the cells were also transduced with the gene for bilirubin-uridine diphosphoglucur onate (UDP)-glucoronosyltransferase, expanded *in vitro* by culturing at 33℃ and finally transplanted into syngeneic animals. Long-term reduction of serum bilirubin in association with the appearance of bilirubin glucuronides ensued[59]. In addition, transplantation of SV40 large T-antigen-immortalised hepatocytes in porto-caval shunted rats and those with surgically-induced ALF has been shown to improve resultant hepatic encephalopathy and survival, respectively[60,61]. Several non-clonal human hepatocyte cell lines successfully transfected with an amphotropic mouse retrovirus containing this gene have remained stable in long-term continuous culture[58]. Nonetheless, the potential clinical applicability of such cell lines has been questioned following reports in experimental animals that hepatocellular carcinoma may develop in hepatocytes expressing the SV40 large-T antigen[62].

Kobayashi *et al*[63] have recently investigated the feasibility of overcoming the otherwise unacceptable risk of tumorigenicity of SV40 large-T-immortalised cells, using a technique of reversible immortalisation in which cells are initially transduced to proliferate *in vitro* prior to the excision of the T-antigen-encoding DNA sequence before transplantation. Specifically, a highly differentiated cell line, NKNT-3, was generated by retroviral transfer to primary adult human hepatocytes of the SV40 large-T gene flanked by LoxP recombination targets, with concurrent expression of a fusion protein conferring sensitivity to gancyclovir. NKNT-3 cells became immortalised without an apparent growth crisis and double in numbers every 48 hours. The immortalising and gancyclovir sensitivity-conferring genes could subsequently be completely excised *in vitro* by transient transduction with a replication-deficient recombinant adenovirus expressing the Cre recombinase, which resulted in Cre/Lox site-specific recombination. After removal of the SV40 large-T gene in this way, NKNT-3 cells stopped proliferating and appeared more differentiated, with nucleus to cytoplasm ratios and cytogranules resembling those of normal primary hepatocytes. While mRNA's for bilirubin-UDP-glucur-onosyltransferase and glutamine synt hetase were each detectable in NKNT-3 cells before reversal of immortalisation, levels increased significantly after excision of the SV40 large-T gene by recombination, with mRNA's for albumin and coagulation factor X becoming detectable only in this latter circumstance.

The efficacy of transplantation of NKNT-3 cells, before or after recombination, was then

assessed in a rodent model of ALF in which 5×10^7 cells, equivalent to approximately 5% of the total number of hepatocytes in an adult rat, were delivered by intrasplenic injection one day prior to 90% hepatectomy under immunosuppression with tacrolimus[63]. Transplanted animals showed substantial improvements in total bilirubin, prothrombin time and blood ammonia levels and survival compared to controls, with a trend towards a particular survival advantage in those animals which received the reverted NKNT-3 cells rather than immortalised counterparts. Taken together, these findings demonstrate the feasibility of controlling the *ex-vivo* expansion of primary human hepatocytes by transfection with the SV40 large-T antigen gene and Cre/Lox-based reve rsible immortalisation, with the removal of the oncogene prior to transplantation promoting a level of differentiated function adequate to sustain short-terms urvival in an experimental ALF setting. Future studies should assess the *in vivo* efficacy of reverted NKNT-3 cells when attached to microcarriers or gel-entrapped as hepatocyte spheroids, modifications which would render them amenable to cryopreservation and storage in cell banks for clinical use as required.

Adjuvant genetic manipulation of transplanted human liver cells may also be used to overcome the current necessity for pharmacological immunosuppression to prevent allograft rejection[64]. This is an important issue since, although reduction or withdrawal of pharmacological immunosuppression in the post-OLT setting has been associated with lower incidences of metabolic, infective and neoplastic complications, side effects once developed such as chronic nephrotoxicity and hypertension may be irreversible[65-69]. One approach to preventing rejection of transplanted cells without the need for immunosuppressive drugs relates to the use of cells previously transfected with the adenoviral E3 gene, which encodes several proteins that are known to inhibit host T cell reactivity[70,71]. Preliminary data in rodents suggests that these proteins have the capacity to protect transplanted hepatocytes from rejection. In particular, Brown-Norway hepatocytes expressing E3 proteins persisted for up to 6 weeks following transplantation into allogeneic Gunn rats, as reflected by reduced serum bilirubin levels, while no reduction in this parameter, indicative of rapid allograft rejection, occurred in control Gunn rats receiving a comparable number of untreated Brown-Norway cells[72]. It remains to be determined whether strategies designed to induce tolerance that are currently under investigation or consideration in the post-OLT situation, such as the adjunctive infusion of donor-derived bone marrow or soluble class I antigens, the use of anti-CD4, anti-CD25 and anti-CD54 monoclonal antibodies, blockade of the CD28-B7 T cell co-stimulatory pathway with CTLA41g, pre-treatment of the recipient with IL-10 or even the withholding of all immunosuppression for the first 24 to 48 hours[73-80], are of relevance when hepatocytes only, rather than with additional non-parenchymal cells and donor-derived leukocytes (as with OLT), are transplanted.

Another potential clinical application of gene therapy in the liver failure setting does not involve the transplantation of liver or other cells, but rather the repeated *in vivo* transfection of skeletal muscle with the gene for human HG F. Ueki *et al*[81] recently reported that such an approach in a rodent model of cirrhosis, induced by repeated administration of dimethylnitrosamine, led to significantly increased plasma levels of human as well as endogenous rat HGF and increased tyrosine phosphorylation of the HGF receptor. Inhibition of fibrogenesis and hepatocellular apoptosis were noted, possibly consequent to reduction in levels of TGF-β1. Gene transfer into the rats' skeletal muscle was achieved using weekly injections of liposomes containing the haemagglutinating virus of Japan and human HGF cDNA inserted into the EcoRI and NotI sites of the pUC-SRa expression vector. Survival of the animals was significantly improved compared to that in phosphate buffered saline-treated controls, with the cirrhotic lesion completely reversed in all transfected animals within 50 days of the toxic insult. Such HGF gene therapy would seem less likely to have substantial impact in ALF, since plasma HGF levels are already substantially elevated in this circumstance[82, 83]. The prospect of maximising liver regeneration via gene therapy in this latter situation will likely depend, in the first instance, on a more comprehensive understanding of both the expression of cell surface receptors for stimulatory and inhibitory growth factors and the integrity of down-stream effector and adaptor mechanisms, allowing targeted over-expression or inhibition, respectively, of key elements in these signalling pathways.

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