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ALLOGENIC HEPATOCYTE TRANSPLANTATION INIMMUNOMODULATED LEWIS RATS WITH ACUTE LIVER INSUFFICIENCY FOLLOWING HEPATECTOMY

By

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Abstract

Orthotropic Liver transplantation (OLT) is a conventional management for end-stage acute or chronic liver insufficiency, but the shortage of donor organs continues to be the restrictive factor throughout the world. Hepatocyte transplantation (HCTx) might be the promising treatment for several liver diseases and can be used as a "bridge" to OLT. Hepatocytes transplantation can protect and even save human lives, its' applicability remains limited by the large deficiency of liver organs and hepatocytes (HC), and cellular loss after engraftment. Host elimination of grafted cells is called Early Graft Dysfunction.

This study was developed for an efficient protocol of HCT. Several conditions have been met in order to achieve a high yield of harvested viable HC, overcome the detached-cell apoptosis, attenuation of innate immune reaction against transplanted cells and a receptive cell environment. HC were isolated from Lewis rats (n=8) weighing 250gm, by the 2 step collagen a seper fusion technique, and bone marrow cells (BMCs) were obtained from the rats tibia and femur by centrifugation in a buffer solution. The mean viability of harvested HC and BMCs were 90% and 95% respectively.

To minimize the rejection of HC, Lewis rat recipients (n=14) weighing 250gm, were irradiated with 6 Gy and received 0.1 mg of anti-aisle GM1 antiserum intravenously as immunosuppressive drug. The isolated HC were intra-splenically transplanted and 10^7 bone marrow cells were injected in a penile vein into the recipients on the third day. Simultaneously, 70% hepatectomy and ligation of common bile duct were done. Thirty days later; the grafted spleen had areas with external appearance of a normal liver in ten out of 14surviving rats (71%). Hematoxlin and eosin (H & E) staining of sections from these fragments showed sinusoids and portal areas, an evidence of successful hepatocyte engraftment and bile canaliculea formation. Large number of HC clusters of 15 to 20 cells and 2 to 4 distended small bile canaliculea were seen per50 HC. The intrasplenic route for transplanting freshly isolated HC in an immune-compromised animal model was found to give good results regarding cell engraftment and tissue formation.

Keywords: Hepatocytes transplantation (HCTx), intra-splenically transplantation, orthotropic liver transplantation (OTL), rats, bone marrow cells (BMCs), hepatectomy.

Introduction

Despite the progress in OLT, the deficiency of suitable and undamaged organs remains unsolved. This imposes financial burden on the health service on one hand and threatens the patient's life on the other hand. Approximately 80% of patients die before obtaining a life-saving transplant.

In the United States, the United Net-work for Organ Sharing (UNOS) waiting list candidates, as of August 2011, had 111,825 patients. Sadly, about one person dies every 80 minutes while awaiting an organ. Direct

(HCTx) or hepatocytes in an Extracorporeal Mechanical Support Systems, have the potential to either serve as a bridge to OLT or prolong survival to allow recovery of the acutely injured liver (Nyberg *et al,* 1992). The role of (HCTx) in chronic liver disease is less certain. Preliminary, animal and human studies have shown improvement in hepatic encephalopathy induced by portocaval shunts (Ribeiro *et al,* 1992). IL-I8 gene-modified HC intrasplenically transplanted into mice could effectively reverse schistosomal hepatic fibrosis (Zhang *et al,* 2001). Hepatocytes transplantation has successfully treated congenitally hyperbilirubinaemic Gunn-rat deficient in bilirubin-UDP-glucuronyl transferees, the Nagaseanal buminaemic rat, the Watanabe heritable hyperlipidaemic rabbit deficient in low-density lipoprotein receptors and the Long-Evans Cinnamon rats modeling Wilson's disease (Dixit *et al,* 1990; Michael *et al,* 1993; Wilson *et al,* 1991; Irani *et al,* 2001).

Hepatocytes transplantation can protect or even save human lives; still its use was limited by the great lack of organs and HC for transplantation and the cellular loss after engraftment (Han *et al,* 2009).

Most of the intact and active transplanted cells disappear, a phenomenon called "Early Graft Dysfunction". After HC isolation, the death receptors, CD95/Fas and the TNF alpha receptor, leads to anoikis and cellular apoptosis (Zvibel *et al,* 2002). Caspases3, 6 and 7 are activated by Capsase 8 and enhance the death signals. Anoikisisal so caused by disruption of the survival signals, normally induced by attachment to extracellular matrix through integrins and by Growth Factor Receptor signaling. The Hepatic Growth Factors give resistance to anoikis induced by a variety of injuries, and are capable to shield cells from apoptosis. However, apoptosis of HC 2to4 hours after isolation did not exceed 5% of cells (Fox-Marsh and Harrison, 2002; Olszewski *et al,* 1994).

Grafting cells creates a wound by necessity that instantly attract platelets, granulocytes, natural killer cells (NK), macrophages, CD4+ and CD8+ T-cells and later clotting factors and complement. The ultimate early phase of wound healing put away the graft and is independent of the all antigen stimulation (Olszewski *et al,* 1997; Gupta *et al,* 1999). This process takes place intravascularly or in the spleen, liver, subcutaneous tissue or peritoneal cavity.

Blocking HCc adheres with monoclonal antibodies lowers the level of HC cytotoxicity by Leukocytes and serum factors *in vitro* (Olszewski *et al,* 1998). On the other hand, deactivation of serum complement does not decrease the cytotoxicity rate and adding anti-asialo GM1 antiserum (eliminating NK cells) to the mixed HC-leukocytes culture, does not decrease the leukocyte cytotoxicity level (Olszewski *et al,* 2002).

It was reported that blood granulocytes and monocytes elimination by non-lethal whole body irradiation, increased the survival rate of intravenously transplanted HC and non-lysed HC could be found in the lungs and spleen hours later (Olsze- wski *et al,* 2001). The liver thought as an optimal site for HCTx; showed extremely low HC graft survival rate, however, HC were well engrafted when injected into the splenic pulp as they were entrapped in the sinusoid and vascular spaces (Akhter *et al,* 2007).

The aim of this experimental study was to test several laboratory conditions to achieve a high yield of harvested viable hepatocytes from donors, a fertile environment in the recipient for HC proliferation.

Materials and Methods

Several conditions have been met in order to achieve a high yield of harvested viable HC, overcome the detached-cell apoptosis, attenuation of innate immune reaction against transplanted cells and a receptive cell environment as described (Olszewski *et al,* 2002). All experiments were carried out according to the Ethical Rules and Regula-

tions of the Polish Academy of Science, for handling laboratory animals.

Isolation of hepatocytes and bone marrow cells: Hepatocytes were isolated from six months old male Lewis rats (n= 8) weighing 250gm by using the Klauning *et al.* (1981), modification of the two step collagenase perfusion technique according to Berry and Friend(1969).The portal vein was cannulated and the liver in situ perfused, with 500 ml of Krebs's Ringer Buffer "KRB" (137 mmol NaCl, 5.3 mmol KCl, 0.8 mmol Mg $SO_4.7H_2O$, 0.4 mmol Na_2HPO_4, 0.4 mmol KH_2PO_4, 5.5 mmol glucose, and 5 mmol HEPES) to wash blood vessels and hepatic tissue, then, liver digested by collagenase buffer (100 ml of KRB, 0.025gm $CaCl_2$, and 0.05gm collagenase), to disperse connective tissue components. The liver was then mashed using a sterile metal comb, filtered through a sterile stainless steel mesh and thoroughly washed in a buffer by the low speed centrifugation. Supernatants were discarded and the cell pellets re-suspended in 50 ml of washing buffer. Cell count was determined by a haemocytometer and cell viability checked by mixing equal volumes of cell suspension with
0.4% trypan blue dye. Cells having viability >75% were used for transplantation on the same day.

BMCs were obtained from the shafts of tibia and femur of Lewis rats by low speed centrifugation at room temperature. The supernatant was discarded and the cell pellet was incubated on ice with 2 ml of lysing buffer solution to remove red blood cells. Cells were washed using 10 ml RPMI 1640 medium, supplemented with penicillin, streptomycin and fungizone (10,000 units/ml, 10,000 µg/ml and 250 µg/ml respectively) and re-suspended in complete RPMI 1640 medium containing 5% fetal calf serum (Gewartowska and Olszewski, 2007). The cell viability was also checked by 0.4% trypan blue dye.

Transplantation: To compromise the immune system of the animals, Lewis rats (n= 14) weighing 250 gm were subjected to sublethal dose of irradiation (6 Gy).

Two days later, rats had 0.3 ml (0.1 mg) of anti-asialo GM1 antiserum in saline to eliminate NK (Gewartowska and Olszewski, 2007). On the 3rd day, rats were anesthetized with diethyl ether alcohol and 0.3 ml heparin (5000 U/ml) followed by BMCs (10^7 cells/0.3 ml saline) were injected into the penile vein to reconstitute the cellular reserve of the animal. Next, the common bile duct was ligated, a 70% hepatectomy performed and the spleen was simultaneously engrafted with 10^7 allogeneic hepatocytes in 0.3 ml saline, with temporary occlusion of the splenic pedicle (Olszewski *et al,* 2002). Thirty days after transplantation, splenic specimens were fixed in 10% neutral buffered formalin solution for 24 hours and processed into paraffin blocks and stained with H & E for histological assessment.

Results

Harvested HC count was >200 x10^6 cell in 18% (n=3), 200 to 100x10^6 cells in 53% (n=9) and <100x10^6 cells in 29% (n=5) of rats. On the other hand, cell viability was 75 to 93% in 82% (n=14) of rats (Fig.1).

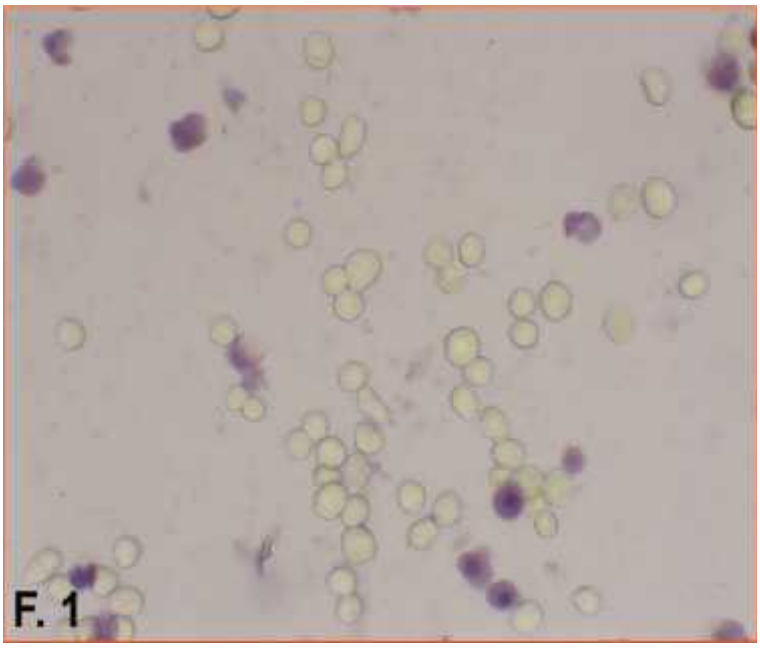

Figure 1: Numerous viable HC excluding trypan blue dye and few darkly stained dead cells (X40).

BMCs count isolated was >200 x 10^6 cells in 50% (n= 7) and cell viability ranged from 83% to 95% of rats. Out of fourteen rats transplanted, 71% (n=10) survived. The spleen had an external appearance of irregular normal liver lobules. The pathological study of spleen sections with H & E staining indicated irregular liver lobules formed of sinusoids and portal areas. HC appeared as sheets and clusters of polygonal epithelial cells, approximately 40 µm in diameter, with eosinophilic cytoplasm and basophilic stippling. They have a single or double centrally located large nucleus (Fig. 2 A). Bile ductules appeared as wide distendedspaces surrounded by cholangiocytes among the lymphoid tissue of the spleen (Fig. 2 B). HC clusters of 15 to 20 cells and two to four bile canaliculae were seen per 50 HC.

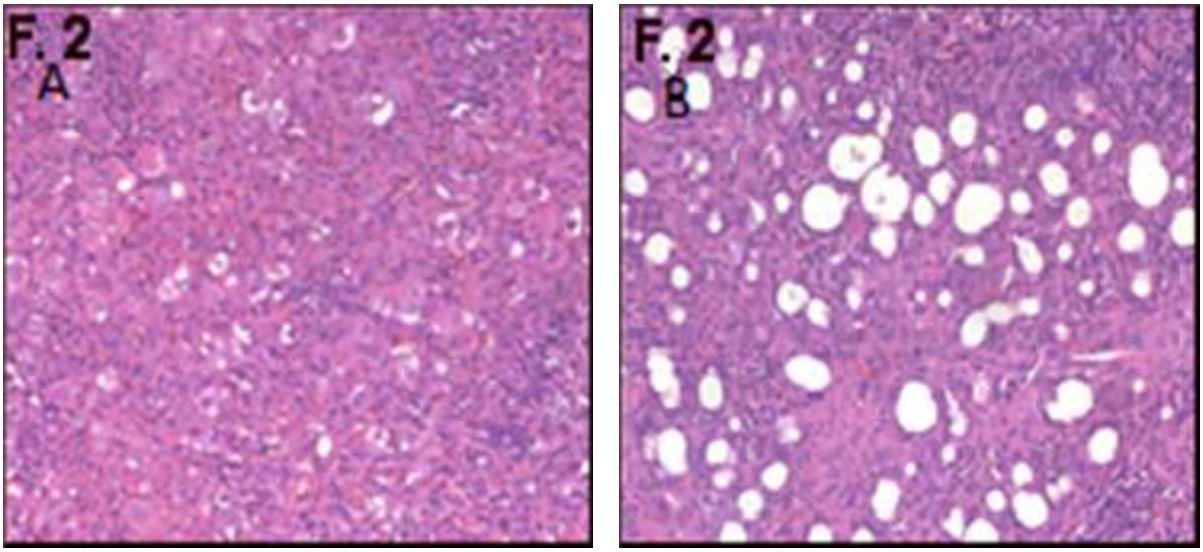

Figure 2: Histopathology of spleen sections (H & E). Clusters of HC (A, X10) and bile ductules surrounded by cholangiocytes (B, X20) among lymphoid tissue of spleen.

Discussion

The liver was among the first organs considered for isolated cell transplantation (Weber *et al,* 2009). Isolated HCTx could potentially be used for the treatment of liver failure and innate defects of liver-based metabolism (Minato *et al,* 1984). The first HCTx was performed to treat the Gunn rat, the animal model for Crigler-Najjar syndrome (Groth *et al,* 1977).

Transplanted cells require a direct contact with stromal cells, the producers of ground matrix with adhesion molecules and where cytokines, chemokines and growth factors are stored. The cells need adequate blood perfusion for nutrients and specific systemic regulatory factors, e.g. insulin, glucagon, thyroxin…etc.

Moreover, for transplanted cells proliferation, an adequate space and fibrillar carcass for attachment are indispensable (Si-Tayeb *et al,* 2010; Demetriou *et al,* 1986; Ohashi *et al,* 2001). Grafted HC have a very short survival, largely because of the inadequate initial cell mass (Si-Tayeb *et al,* 2010). The approximate number of HC needed and an effective extra-hepatic site for hepatocyte transplantation have still not been identified.

Several sites for grafting, including the liver and spleen (Si-Tayeb *et al,* 2010; Kusano and Mito, 1982; Matas *et al,* 1976), the peritoneal cavity, pancreas, lung parenchyma, under the kidney capsule and thymus were studied (Demetriou *et al,* 1986; Jaffe *et al,* 1988; Then *et al,* 1991; Ricordi *et al,* 1989). In cases of metabolic liver disease and acute liver failure, in which the liver architecture is intact, the presence of physiological matrix and the availability of portal blood supply make the liver the optimal site for hepatocytes transplantation (Weber *et al,* 2009). However, when the hepatic architecture is modified, as in cirrhosis, transplanting HC into the portal vein may result in prolonged exaggeration of portal hypertension, embolization of the cells to the lungs (Strom *et al,* 1999) and it is doubtful that the transplanted HC can function for a sustained period of time (Mito *et al,* 1992).

Transplanting HC into the spleens of rats with decompensated liver cirrhosis can improve liver functions and prolong survival. It significantly improved or corrected prothrombin time, serum albumin and bilirubin levels (Soltys *et al,* 2010; Nagata *et al,* 2003). Some HC migrated to the liver via the portal venous system. Several authors, also, used the spleen as a route for transplantation (Nagata *et al,* 2003).

Ectopic sites for HC engraftment, particularly the peritoneal cavity and subcutaneous tissues, have been investigated by Gewartowska and Olszewski (2007), but these sites do not immediately supply the transplanted cells with oxygen and nutrients. The renal capsular space has, also, been used, but it can accommodate only a small number of liver cells (Weber *et al,* 2009). Injecting cells in several ectopic locations is believed to enhance the therapeutic potential of cell therapy, because it enlarges the tissue mass grafted, and helps immunological tolerance.

The allogeneic HC transplanted into the thymus of animals, without immunosuppression, expressed specific liver functions for more than 42 days which could be very useful in inborn disorders of metabolism (Mula *et al,* 2008).

The present data show that the two step collagenase perfusion technique used for HC and the BMCs separation method gave excellent results regarding cell number and viability. The authors transplanted HC on the same day of their isolation, although Wang *et al*. (1995) and Jiang *et al* .(1997) believed that HC might be injured by the process of isolation and purification and need to be cultured with trophic factors, as insulin, or be grafted few days before inducing liver insufficiency by hepatectomy. The surgically induced acute hepatic failure by 70% hepatectomy and ligation of the common bile duct, as used by Emond *et al.* (1989), Eguchi *et al*. (1996) and Wesolowska *et al.* (2004) was also done in this exper-

iment. Unlike the drug-induced liver insufficiency, this method is simple, highly standardized and does not confuse the pathophysiologic changes (Gewartowska and Olszewski, 2007).

The autologous HCTx initiates non-specific humoral and cellular cytotoxic reactions, by granulocytes and macrophages against the transplanted cells. In case of allogeneic or xenogeneic transplantation, the natural antibodies and tissue macrophages also attack and destroy the transplanted cells. Shultz *et al.* (2007) and Hughes *et al*. (2008) found that most of the transplanted HC died within 5 days without immunosuppression, although immunosuppression by rapamycin had a deleterious effect on the engraftment and proliferation of transplanted HC (Weber *et al,* 2009).

The present study confirmed that temporarily depleting granulocytes and monocytes by non-lethal whole body irradiation, and eliminating NK by anti-asialo GM1 antiserum, increased the survival of transplanted HC (Wesolowska *et al,* 2003; 2004). The transplanted cells showed evidence of successful HC engraftment and bile canaliculi formation in 70% of the immunocompromized recipient rats. This was clearly observed in H & E stained sections of the recipient rats' spleens. These results agreed with the data of Malhi and Gupta (2001) but cell function must be tested before it can be stated that liver tissue repopulation potentially eliminates the requirement for OLT.

Acute liver failure after a major liver resection has a high incidence of morbidity and mortality, especially in patients with liver diseases. OLT, suggested as the most effective treatment for patients with acute liver failure, has technical and logistic obstacles. Unfortunately, till today (HCTx) in human with end-stage cirrhosis resulted in only improvement in some parameters of liver function (Lee *et al,* 2004; Khan *et al,* 2004).

Designing a method for isolating and engrafting HC from liver organs unsuitable for transplantation is needed and may ease the problem of organ availability to transplantation and so much so in countries where cadaveric organs transplantation is not permitted (Dhawan *et al,* 2006; Nussler *et al,* 2006).

Foot Note

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