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Experimental transplantation of hepatocytes in cases of toxic acute liver failure. An allograft model

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Abstract The aim of this experimental study was to modify a rat liver-cell harvesting technique and to evaluate the efficacy of allogeneic liver cell transplantation (Tx) using cyclosporin A immunosuppression in rats with N-dimethylonitrosamine (N-DMNA)-induced acute liver failure (ALF).
Twenty male Wistar rats, weighing 190–320 g, were used as donors. Hepatocytes were harvested by the use of a modification of the Seglen portal vein collagenase perfusion technique (type V/1.3 mg/ml), which resulted in the isolation of a mean of 8000 viable clusters of hepatocytes per donor (viability was measured using the trypan blue exclusion test). The male Lewis recipients and controls received 20 mg/kg N-DMNA IV, and were then divided in three groups. Group 1 ($n = 5$) received no treatment, group 2 ($n = 10$) received 5000 clusters of freshly isolated hepatocytes (FIH) in the spleen 24 h after the administration of N-DMNA- and group 3 ($n = 10$) received 5000 clusters of FIH beneath the renal capsule, 24 h after the administration of N-DMNA. All groups were treated with cyclosporin A 20 mg/kg per day IP. SGOT and bilirubin values were measured and all surviving rats were sacrificed on day 14. All rats in group 1 died of histologically confirmed liver necrosis within 72 h. The 14-day survival was 60% in group 2 and 50% in group 3. The post-Tx SGOT values reached their maximum on days 3–4 (group 2, mean 754; group 3, mean 529) and were only slightly elevated on day 14 (group 2 = 75, group 3 = 48). The post-Tx bilirubin values reached their maximum on days 3–5 (group 2 = 1.1, group 3 = 1) but failed to return to normal until day 14. Autopsy and histological examination of the surviving animals showed well-preserved hepatocellular spherical aggregates in the spleen and hepatocellular "cords" in the kidney accompanied by signs of regeneration of the native liver. We concluded that the hepatocyte Tx in a rat experimental allo-Tx model improved the survival rate and the SGOT values in cases of toxic ALF. Survival rates between the two different sites of Tx were similar.

Key words Acute liver failure · Cyclosporin · Experimental transplantation · Hepatocyte transplantation · Hepatocytes · N-dimethylonitrosamine

Introduction

Since it was first developed in the 1970's [1], hepatocyte transplantation (HTx) has generated increasing interest as an alternative to whole liver Tx in cases of acute fulminant liver failure (ALF) where a metabolic support system is needed until the host liver regenerates or a suitable liver graft is available. HTx has the advantages of minimal intervention, major cost-effectiveness and preservation of the native liver. In addition, hepatocytes (HCs) are isolated in large numbers from a single donor, can be cryopreserved [2] and are easy and safe to transplant [3]. Although the spleen is the best-studied organ for homing HCs in rats, other sites (peritoneal cavity, kidney, lung, pancreas, dorsal fat pad, etc) have been proposed in a variety of enzyme deficiency, toxic acute, anoxic acute and chronic hepatic failure models [3–5]. The present study was the first attempt to experimentally isolate and transplant liver cells in Greece. The aim of our study was to assess the survival rate and liver function in cases of N-dimethylonitrosamine (N-DMNA)-induced toxic ALF when large numbers of allogeneic HCs (5000 clusters) were transplanted across a major histocompatibility barrier (Wistar to Lewis) using cyclosporin A immunosuppression. We also aimed at comparing survival and hepatic function between two different sites of HTx; spleen vs. kidney subcapsular. A modification of the Seglen liver cell harvesting technique is also proposed.

Materials and methods

Animals

Adult male Wistar and Lewis rats weighing 190–320 g were obtained from Pasteur Institute, Athens. Rats were acclimatized to our laboratory conditions for 1 week prior to use in experiments. They were housed individually in stainless steel cages at a constant temperature (29 °C) and a 12-h day/night cycle. Rats ate commercial rat chow and had water ad libidum.

Induction of hepatic failure

All Lewis rats received 20 mg/kg N-DMNA IV on day 0 of each experiment. This dose has been shown to result in reproducible, potentially reversible hepatic necrosis and high mortality rates.

Isolation of hepatocytes

For isolation and preparation of hepatocytes, a modification of the Seglen technique was used. The ten Wistar rats were anaesthetized with pentothal-diazepam and a formal laparotomy was carried out. After systemic heparinization, the portal vein and suprahepatic inferior vena cava were cannulated while the infrahepatic inferior vena cava, hepatic artery and bile duct were ligated. The liver was perfused with 100 ml of Ca^{++}-free Hanks balanced salt solution (HBSS) at a flow rate of 10 ml/min. Collagenase type V/1.3 mg/ml with 5 m*M*/l $CaCl_2$ added to HBSS solution was similarly perfused and recovered via the supra hepatic inferior vena cava cannula and reperfused for 15 min at a rate of 10 ml/min. The liver was then removed and placed in a sterile petri dish on ice, cut in small parts and incubated in collagenase solution at 37 °C for 45 min. The remaining liver particles were then gently massaged through a metal sieve (pore size 400 μm) in order to take off the liver connective tissue. The cells were washed three times in Hanks solution with 5% newborn calf serum. The suspension was centrifuged, and the cell hepatocytes recovered were counted. Viability was assessed by the trypan blue exclusion test.

Transplantation

Lewis rats were randomly assigned to one of three groups: group 1 ($n = 5$) received no treatment, group 2 ($n = 10$) received a transplantation of 5000 clusters (approx. 2×10^8 cells) of freshly isolated hepatocytes into the spleen 24 h after the administration of N-DMNA and group 3 ($n = 10$) received 5000 clusters of freshly isolated hepatocytes beneath the renal capsule, 24 h after N-DMNA administration. All rats were transplanted under ether anaesthesia. The cell suspension was diluted in 0.5 ml of Hanks and was injected using a 27 gauge needle. During the intrasplenic injection the splenic blood flow was occluded with clamps. In both transplantation sites (spleen and kidney) leakage was prevented with microsurgical sutures at the point of injection. All recipients (groups 2 and 3) and controls (group 1) were started on daily cyclosporin A intraperitoneal immunosuppression on day 0 of transplantation (day 1 after N-DMNA) as follows: 20 mg/kg on days 0–4 and 10 mg/kg on days 5–14. Blood samples were collected daily in order to assess the bilirubin and SGOT levels. On day 14 after the ALF induction, all surviving animals were sacrificed by an overdose of anaesthetic agents.

Histology

All rats underwent autopsy and removal of their liver, spleen and kidneys. Specimens were fixed in 10% neutral buffered formalin and examined under light microscopy.

Results

Number and viability of isolated hepatocytes

A mean of 8000 clusters of viable hepatocytes were isolated per donor. The viability of the isolated hepatocytes was greater than 90%, measured with the trypan blue exclusion test. Microscopic observation showed that contamination from red blood cells was less than 2%.

Table 1 Mean serum SGOT and bilirubin (Bili) concentrations on days 3, 5, 7 and 14. Cyclosporin A was administered daily to all groups

Group	Treatment		Mean values							
	Day 0	Day 1	Day 3		Day 5		Day 7		Day 14	
			Bili	SGOT	Bili	SGOT	Bili	SGOT	Bili	SGOT
1 ($n=5$)	N-DMNA	–	2.7 (3)	> 3000 (3)	–	–	–	–	–	–
2 ($n=10$)	N-DMNA	Hepatocytes intrasplenic	1.1 (9)	754 (9)	0.6 (8)	450 (8)	0.09 (6)	144 (6)	0.03 (6)	75 (6)
3 ($n=10$)	N-DMNA	Hepatocytes beneath renal capsule	1.0 (8)	529 (8)	0.5 (6)	391 (6)	0.10 (6)	219 (6)	0.04 (5)	48 (5)

Survival rate

All rats in group 1 died of acute hepatic failure within 72 h after the administration of N-DMNA. In group 2 (intrasplenic HTx) 60% of rats (six rats) survived until day 14 when the experiment was terminated. One rat died on day 2, one on day 3 and two rats died on day 5. In group 3 (renal subcapsular) 14-day survival was 50% (five rats). Two rats died on day 3, one on day 4, one on day 5 and one on day 10. The mean survival in group 2 was 9.9 days while in group 3, it was 9.5 days. The difference in the mean survival and the 14-day survival rates between groups 2 and 3 was not statistically significant ($P > 0.05$). The difference in survival between groups 1 and groups 2 and 3 combined was statistically very significant.

Biochemical data

Table 1 summarizes the mean bilirubin and SGOT values during the 14-day period of experimental observation, in groups 1, 2 and 3. Rats that did not survive the whole 14 days, in groups 2 and 3, were excluded. After N-DMNA administration, rats responded with a large increase in transaminases and bilirubin. In group 1, mean SGOT values increased to greater than 3000 IU/l and mean bilirubin values to 2.7 mg % on day 3. In groups 2 and 3, the SGOT values reached their maximum on days 3–4 (day 3: group 2 mean, 754 IU/l, group 3 mean, 529 IU/l) and were only slightly elevated on day 14 (group 2 mean: 75, Group 3 mean, 48). The bilirubin values reached their maximum on days 3–5 (day 3: group 2 mean, 1.1 mg %, group 3 mean, 1 mg %) but failed to return to normal until day 14 (group 2 mean, 0.03 mg %, group 3 mean, 0.04 mg %). Normal values were determined by repeated measurements in healthy controls as follows: SGOT, less than 70 IU/l, less than 0.01 mg %. The elevation of SGOT and bilirubin values in groups 1, 2 and 3 was statistically significant compared to pre-N-DMNA values. Mean SGOT and bilirubin values were not significantly different between groups 2 and 3 on any day of observation. The decline in bilirubin and SGOT after day 5 in groups 2 and 3 was statistically significant compared to pre-Tx values.

Autopsy histology

Mortality after administration of N-DMNA correlated with the extent of liver damage as assessed by histological examination. Intraparenchymal haemorrhage with "congestive" centrolobular hepatic necrosis was severe in all rats in group 1 and in those rats from groups 2 and 3 that died on days 2, 3, 4 and 5. The livers of rats surviving 14 days showed marked signs of recovery and restoration of the hepatic architecture. All rats in group 2 had spherical aggregates in the red pulp of the spleen without reconfiguration of the normal liver structure. In group 3, single layers or clusters of HCs were detected close to the renal capsule or in the renal pelvis (one case). Cord formation was more frequent in group 3. Four cases of ischaemic necrosis and degenerative alterations of HCs were noted in 14-day survivors while two cases of rejection (in a 10-day survivor and a 14-day survivor) were evident (appearance of giant cells, atrophy of HCs, phagocytosis).

Discussion

Hepatocyte transplantation for ALF has been extensively investigated in rat models and seems to be an established method for increasing survival and improving liver function. We chose the N-DMNA-ALF model because it requires minimal intervention prior to HTx (no laparotomy needed) and results in very high ALF rates.

Both the intrasplenic and the renal HTx proved to be effective in terms of survival and improvement in liver function tests. The use of cyclosporin A, permitted HTx accross a histocompatibility barrier with a low incidence of rejection (two cases). Since no evidence of technical complications was found, we concluded that early mortality (days 2–5) was possibly due to primary nonfunction of the injected cells. Biochemical data and histological findings suggested considerable recovery of liver function after day 5. Liver regeneration was very evident on day 14. No difference between the two HTx sites was noted. However, in these studies there was wide fluctuation in the level of response to treatment and large numbers of animals together with complex statistical analysis are needed to establish all possible differences in survival and biochemical function between intrasplenic and kidney HTx. Intrasplenic hepatocytes have also been proved to secrete albumin, conjugate bilirubin, excrete ^{99}Tc-HIDA and contain P-450, but these properties were not studied in this protocol. There is still controversy about the number of HCs that need to be transplanted, about the mechanisms of the metabolic support provided by HTx and the liver regeneration that occurs, possibly regulated by various hepato-proliferative factors [2–5].

Future clinical prospects of HTx include correction of enzyme deficiency in humans, application as a possible adjunct or alternative to whole organ Tx, gene replacement treatment and development of artificial liver systems [5–7]. The high incidence of hepatitis B cirrhosis and the severe shortage of liver donors in Greece make HTx an appealing future solution for temporary metabolic support of patients awaiting transplantation or retransplantation after rejection. Newly developed methods of human HC isolation will make a major contribution towards the future clinical use of HCs ([7] and work recenty completed by our group).

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