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Cryopreserved Fetal Liver Cell Transplants Support the Chronic Failing Liver in Rats With CCl_4-Induced Cirrhosis

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Hepatocyte transplantation is a promising method for supporting hepatic function in a broad spectrum of liver diseases. The aim of this work was to test the efficacy of human fetal liver cells to support the chronic failing liver in an experimental model of carbon tetrachloride (CCl_4)-induced cirrhosis in rats. Liver cirrhosis was induced by intraperitoneal administration of CCl_4 at a dose of 0.2 ml (50% v/v solution)/100 g body weight, twice a week for 3 months in rats. Ten days after stopping CCl_4 administration (experimental day 0), rats received intrasplenic injection of cryopreserved fetal liver cells (FLC, 1×10^7 cells in 0.3 ml medium). As a cirrhotic control group, CCl_4-induced cirrhotic rats were used with intrasplenic injection of an equal volume of medium alone. Animals were sacrificed on experimental day 15. Human fetal liver cell transplantation almost completely prevented the death of cirrhotic animals during the 2 weeks after treatment, while high ongoing mortality was seen in the cirrhotic control group. Cell transplantation into the spleen normalized total bilirubin and TBARSs levels and increased albumin levels in blood serum, as well as restoring mitochondrial function and liver detoxification function (assessed by cytochrome P450 contents and activity) compared with the activities seen in the cirrhosis control group. In parallel with this restoration of biochemical and functional liver indices, morphological patterns of liver recovery or regeneration after liver cell transplantation were demonstrated in day 15 samples by light microscopy. These were absent in the group that had received only medium alone.

Key words: Fetal liver cells; Human; Cryopreservation; Liver cell transplantation; CCl_4-induced cirrhosis; Rat liver function

INTRODUCTION

Liver transplantation is considered today as the main tool for restoration of liver function in patients with acute liver failure, end-stage chronic liver disease, as well as in inherited metabolic liver disease (31). The scarcity of organs has led to a search for other means of correction of such metabolic defects. During past the decade several studies were published using transplantation of isolated hepatocytes in animals with liver failure or with enzyme deficiencies (8,14,33), and in a limited number of clinical situations. In models of isolated hepatocyte transplantation, the site of cell transplantation has been discussed. It has been shown (15) that only intrasplenic (not intraperitoneal) transplantation of hepatocytes could significantly improve survival and the physiologic abnormalities associated with decompensated cirrhosis and liver failure. Hepatocyte transplantation into the spleen has also been used for the treatment of enzyme deficiency disease (39) and acute severe liver failure in the rat (13).

Nevertheless, the clinical application of the procedure is rare (27) because of limited source of hepatocytes for isolation and concern about the duration of function after transplantation as allografts. Another promising possibility within the field of hepatocyte transplantation, which practically has not been explored despite the theoretical advantages, is fetal liver cell (FLC) transplantation. Fetal cells, as stem cell progenitors, present a number of theoretical advantages. They are less immunogenic than adult cells and, therefore, control of rejection may be easier. They have a high proliferative capacity, meaning that a smaller number of cells could replicate to provide the necessary hepatic function, and because they are less differentiated, they could specialize to compensate for a variety of deficient metabolic pathways after transplan-

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tation to correct a variety of liver diseases. The smaller size of FLC could be decisive in enabling them to rapidly migrate directly into the liver. An additional theoretical advantage might be that FLC could be easier to work with as far as genetic manipulation and storage are concerned. It has been shown that isolated FLC transplanted into analbuminemic rats differentiate to acquire an adult phenotype and present signs of a potential therapeutic capacity (29). However, there are few reports on the function of transplanted fetal liver cells and in these cases FLC for transplantation were obtained from fetuses of the latest gestation term (29). The effect of FLC transplantation on chronic liver failure has not been extensively studied. At one time, human fetal liver of the first trimester gestation contains both hepatic (34,35) and hemopoietic (28) stem and progenitor cells. Recent studies suggest that hemopoietic stem cells may also form hepatocytes in vivo and in vitro (17,37).

The aim of this work was to test the efficacy of human FLC as a xenograft to support the chronic failing liver in rats. For this purpose we used human liver cells of the first trimester gestation, which were transplanted to the rats with CCl_4-induced cirrhosis.

MATERIALS AND METHODS

Adult male Wistar rats weighing 150–180 g were used in our experiments. Animals were kept under standard conditions with free access to food and water. Animals were housed and treated as dictated by the guidelines of the Ukraine Academy of Sciences and The Local Institute Committee on Ethics and Welfare. All surgical interventions in animals, including decapitation, were performed under anesthesia induced by ether inhalation.

Induction of Cirrhosis

Cirrhosis was induced by IP injection of CCl_4 (diluted 1:1 in corn oil) at a dose of 0.2 ml/100 g twice a week for 3 months. Every week the body weight was checked. If it became 5–10 g lower than the initial value, the dose injected was reduced to 0.15–0.1 ml/g weight in agreement with previously published protocols (3). The development of liver pathology was checked by alterations in plasma albumin levels and total bilirubin contents in blood once each month compared to normal animals.

For this study, there were four experimental groups. Group 1 was normal, age matched untreated animals ($n = 8$). Group 2 (confirmed cirrhosis, $n = 8$) was animals studied 10 days after cessation of CCl_4 administration for the previous 3 months, by removal of liver and blood samples under terminal anesthesia. Group 3 was CCl_4-exposed, sham-transplanted rats (rats with CCl_4-induced cirrhosis and receiving intrasplenic injection of 0.3 ml medium alone, $n = 12$). This was performed 10 days after cessation of CCl_4 administration for the previous 3 months. Group 4 was CCl_4-exposed, FLC-transplanted rats (rats with CCl_4-induced cirrhosis and receiving intrasplenic transplantation of 10^7 cryopreserved human fetal liver cells in 0.3 ml medium, $n = 11$). This was performed 10 days after cessation of CCl_4 administration for the previous 3 months.

Preparation of Fetal Liver Cell Suspension

FLC were isolated from human fetuses 8 to 12 weeks old. Fetuses were obtained from routine surgical termination of pregnancy where patients had volunteered to donate fetal tissue (under informed consent for therapeutic use and research as dictated by the Ukraine Ministry of Health). The fetal livers were dissected on ice under sterile conditions and washed three times in Hank's solution. Liver fragments were disintegrated to form a single cell suspension in RPMI-1640 by vibration as described in earlier reports (16,26). Briefly, tissue fragments were placed in a petri dish and divided finely by scissors in a small volume of ice-cold medium comprised of sucrose (250 mM) with 5 mM KCl, 0.8 mM Mg_2SO_4, 0.4 mM KH_2PO_4, 0.4 mM Na_2HPO_4, and 1.2 mM $CaCl_2$ (pH 7.4). The tissue fragments were transferred to a polypropylene tube (diameter 11 mm) containing 1–2 ml of the same ice-cold buffer. Cells were liberated by means of controlled vibrational disaggregation using a motor-driven flat pestle (16,26), in 2 min cycles, with a frequency of 30–50 Hz and a 5-mm distance of travel. The cell suspension was then filtered through a standard blood transfusion filter system. Full details of the method and equipment have been described elsewhere (16,26).

The nucleated cells were counted and diluted with a medium supplemented with antibiotics (penicillin/streptomycin in final concentration of 100 units) to give a final cell concentration of $6–8 \times 10^7$ cells/ml. Cells were slowly mixed with an equal volume of cryopreservation medium containing the same sucrose-based salt solution and 20% DMSO and divided in aliquots of 1 ml in cryotubes and equilibrated with this medium for 20 min on ice (36). They were then cryopreserved by a slow cooling protocol with an initial cooling rate of −1°C per min down to −80°C in a programmable freezing machine and then transferred to liquid nitrogen for periods not less than 2 months. Cells from six preparations were stored and used for transplantation.

Immediately before use, samples were thawed in water bath at 37°C. Recovered FLC showed a mean viability of 68 ± 6% by the trypan blue dye test and were used for transplantation.

Transplantation Procedure

Sham-transplanted cirrhotic animals (group 3) and cirrhotic animals receiving FLC transplants (group 4)

underwent transplantation procedure. Under anesthesia, a small surgical incision was made in the flank of the animal and the spleens were exposed. For group 3, the animals received an intrasplenic injection of 0.3 ml of normal cell cryopreservation medium alone. For group 4, the animals each received an injection of 0.3 ml medium, containing 10^7 FLC into the red pulp of the spleen using a tuberculin syringe, and the incision was repaired by suture.

Two weeks after intrasplenic transplantation, all the animals in groups 3 and 4 were killed by decapitation under anesthesia. Blood was collected and LDH activity, albumin content (Alb) (kit: Sigma, St. Louis, MO, USA), thiobarbituric acid-reactive substances (TBARS), and total bilirubin (TB) content (kit: Vector-Best, Russia) were measured in serum by spectrophotometric means (Cary 50, Australia).

For groups 1 (normal untreated), 3 (sham transplanted), and 4 (FLC transplanted), on removal livers was washed out of blood by venous infusion with Hank's solution, cleaned from adherent debris, and put into cold medium containing 0.3 M sucrose, 10 mM Tris-HCl, 0.5 mM EDTA (pH 7.4). After cooling and weighing, specimens were taken for histology. The remaining liver tissues were gently squeezed through a mesh (1 mm diameter) using a lever press and homogenized in 5 volumes of the same medium in a glass Teflon Daunce handle homogenizer.

Mitochondrial and Microsomal Functions

Both mitochondrial and microsomal functions were studied in samples from the homogenate for groups 1, 3, and 4. The respiratory activities were determined by the standard polarographic method using a Clark-type electrode in a 1 ml cell at 26°C (Rank model 20, UK) in a medium containing 0.25 M sucrose, 10 mM KH_2PO_4, 30 mM Tris-HCl, 0.5 mM EDTA (pH 7.4). A solution of 8 mM succinate was used as a substrate. In 3–4 min after the onset of substrate oxidation, 50 μM 2,4-dinitrophenol (DNP) was added to the cell. Respiratory control index (RCI) was calculated as the ratio of substrate-linked oxygen consumption rates after and prior to DNP addition.

Cytochrome P450 (CYP) in liver homogenate was determined by the spectrophotometric method (22) using the coefficient of molar extinction 10.4×10^3 M^{-1} sm^{-1} on a spectrophotometer Specord UV VIS (Germany). Microsomal *N*-demethylase activity was estimated by formaldehyde formation (24). The molar extinction coefficient was 8.0 mM^{-1} sm^{-1} (21). Aniline hydroxylation was determined by measuring the formation of *p*-aminophenol (11). The incubation medium contained 67 mM Tris-HCl (pH 7.8), 16 mM $MgCl_2$, and 3 mM NADPH. Concentrations of substrates used in the assays were 8 mM aminopirine and 3 mM aniline. Protein concentration was determined by the method of Lowry et al. (19).

Morphology

For histology, liver specimens were fixed in neutral 10% buffered formalin, embedded in paraffin, and stained with hematoxilin-eosin (H&E).

Statistical Analysis

Values are expressed as mean ± SEM. The Mann-Whitney test (for nonparametric values), chi squared test, and Student's *t*-test were used as appropriate. Results were considered significant when $p < 0.05$.

RESULTS

Experimental Cirrhosis

During the formation of the experimental chronic liver pathology there was a mortality of about 40%, which is typical for this model (23). When CCl_4 injection was stopped after 3 months, there was an ongoing mortality with reduction in motional activity and decline in body weight still observed, the color of hair changed, and there was partial hair loss. Most of the cirrhotic animals had dyspepsia, hematuria, and tendency to skin disease and abscesses. These symptoms testified to the serious metabolic derangements in the experimental animals even after cessation of CCl_4 administration.

The morphological confirmation of the extent of liver damage was carried out 10 days after cessation of CCl_4 injection (group 2, confirmed cirrhosis) in all five livers examined. The fixed liver sections of normal untreated rats (group 1) stained with H&E showed normal lobular architecture with central veins and radiating hepatic cords (Fig. 1A). In those animals after prolonged CCl_4 treatment, we observed marked changes in the organ architecture typical of cirrhosis. The cirrhosis was presented as diffuse "piecemeal" active cirrhosis throughout the liver, with disruption of the liver lobules by thin bands of fibrous tissue, presence of inflammatory cells, and small foci of necrosis, particularly in the region of the central vein (Fig. 1B).

In a similar way, all the animals that survived the CCl_4 exposure (groups 2, 3, and 4) showed biochemical evidence of liver damage compatible with the development of cirrhosis. Table 1 shows that total bilirubin concentration (TB) in the serum of animals in group 2 (confirmed cirrhosis) at day 10 after stopping CCl_4 injection was 2.3 times more than the value in normal untreated rats, whereas serum albumin (Alb) concentration was 1.5 time less. The content of TBARS in blood serum increased by twice the normal value (9.6 ± 0.7 μmol/L). Thus, the morphological and biochemical data testified to development of chronic liver pathology corresponding to experimental cirrhosis after CCl_4 administration.

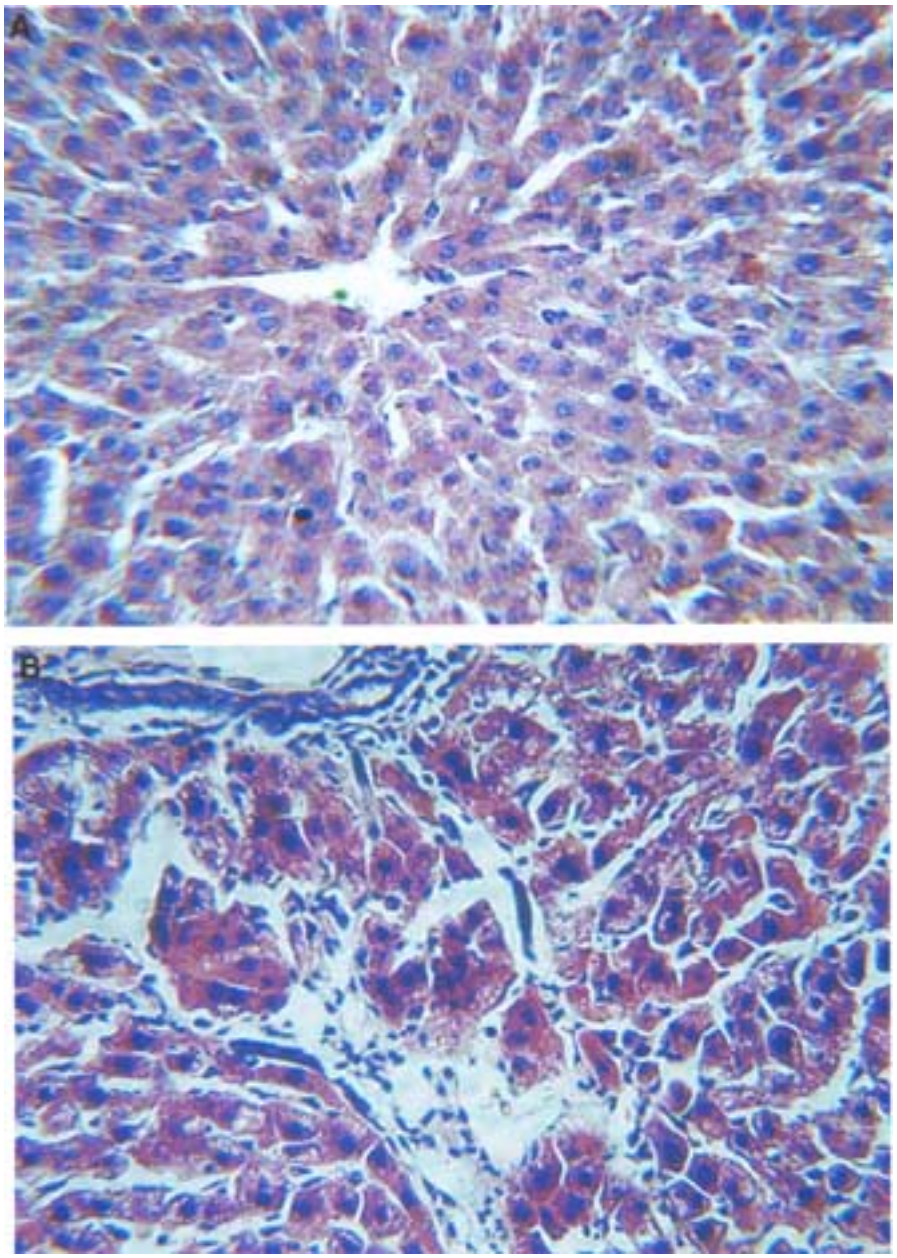

Figure 1. Histological sections (A) of rat untreated liver (group 1) and (B) after 3 months of CCl_4 treatment (group 2, confirmed cirrhosis). Staining with hematoxyline-eosin (original magnification 250×). (A) Normal lobular architecture of liver with central veins and radiating hepatic cord. (B) Prolonged administration of CCl_4 caused severe pathological damage: inflammation, necrosis, and collagen deposition, all characteristic of active cirrhosis.

The Effect of FLC Transplantation on Animal Survival Rate and Liver Morphology

A typical FLC suspension after recovery from cryopreservation is shown in Figure 2. This were seen to be comprised of the expected two-cell populations known to exist in fetal liver: those of hemopoeitic lineage (small cells with darkly staining nuclei) and those of hepatocyte lineage (large cells, with typical single "balloon-shaped" nuclei). The image confirms that the FLC withstood the procedures of isolation and cryopreservation, with little evidence of damage or destruction.

The experimental animals in group 3 (sham transplanted after CCl_4 exposure) and group 4 (FLC trans-

Table 1. Biochemical Indices in Blood Serum of the Animals With CCl_4-Induced Cirrhosis

Group	TB (μmol/L)	ALB (g/L)	TBA-Active Product (μmol/L)
Normal, untreated ($n = 8$)	7.4 ± 0.7	43 ± 3	4.5 ± 0.4
Confirmed cirrhosis ($n = 8$)	17.6 ± 0.9*	28 ± 1*	9.6 ± 0.7*

*$p < 0.05$ versus normal (group 1).

planted after CCl_4 exposure) were under daily observation for 14 days after intrasplenic injection. The recovery period was the most complicated in the sham-transplanted group (group 3), in which five animals died during first week after the operation, leaving the survival rate at 58% (Fig. 3). In the group of animals with FLC transplantation (group 4) only one rat died during the 14 days, and survival rate was 91%. Survival was significantly higher at day 14 ($p < 0.05$) in the FLC-transplanted animals.

Investigation of histological sections of cirrhotic livers in the sham-transplanted group (group 3) showed no major changes in the CCl_4-induced cirrhosis during the 14 days after the sham injection, with all livers again showing active cirrhosis (Fig. 4A), similar to that seen after CCl_4 treatment alone (Fig. 1B, group 2). The disruption of the plates of hepatocytes within liver lobules, inflammatory cell infiltration, and deposition of fibrous connective tissue were all still clearly evident (Fig. 4A). These results confirm that there was no spontaneous regression of the cirrhotic state even after prolonged periods since the last exposure to CCl_4 in the sham-treated animals.

In contrast, investigation of histological liver sections from animals that had received FLC transplantation (group 4) showed morphological changes that pointed to a clear regenerative process. Two weeks after FLC transplantation, the general architecture of the organs remained abnormal. However, there were signs of intracellular and cellular repair of areas of hepatocytes in comparison with the livers from sham-transplanted cirrhotic animals (group 3) (Fig. 4B). Some false segmentation of the liver parenchyma remained, but hepatocytes with normal microscopic features were found throughout the lobules more frequently. There were "typical" liver parenchymal areas formed from such hepatocytes with light cytoplasm and enlarged nuclei containing chromatin and nucleoli (typical of immature or regenerating hepatocytes), but full recovery of normal lobular structure was still not complete. Binucleate hepatocytes were found more often than in the sham-transplanted group (group 3) samples.

The Effect of FLC Transplantation on Blood Serum Indices

One week after the sham transplant operation (week 1), LDH activity was 2.1 times higher and Alb content

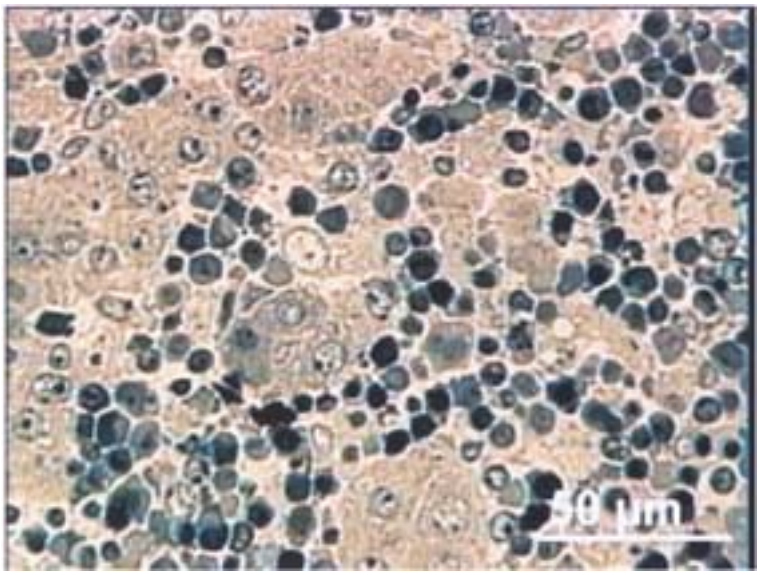

Figure 2. A typical suspension of cryopreserved FLC, after thawing, centrifugation into a pellet, embedding, and staining with toluidine blue dye. The cells are divided into two populations: one comprised of small, darkly stained cells of hemopoietic lineage and the other of large cells, with a single large nucleus typical of hepatocyte appearance.

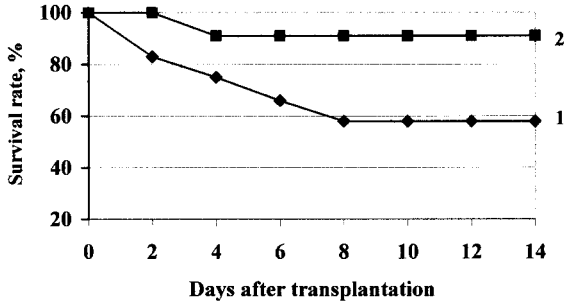

Figure 3. Survival rates in cirrhotic rats (curve 1) during 2 weeks after the sham transplantation (starting number $n = 12$) and (curve 2) during 2 weeks after FLC transplantation (starting number $n = 11$). Survival was higher after FLC at day 14 ($p < 0.05$).

was 1.3 less than seen at day 0 (Fig. 5). These changes were possibly caused by the operative procedure (spleen exposure). However, FLC transplantation (group 4) prevented the increase in LDH activity observed in the sham-transplanted group and resulted in enhancement in Alb content 2 weeks after the operation to 27.7 ± 1.9 g/L. Two weeks after the surgical procedure in the sham-transplanted cirrhotic group, TB level was 1.9 time higher than in the untreated, normal animals (group 1) (Fig. 6A). FLC transplantation (group 4) resulted in decrease in TB level to 9.6 ± 0.8 μmol/L.

Two weeks after the operation, the TBARS content in blood serum in the sham-transplanted cirrhotic group (group 3) reached 6.69 ± 0.16 μm/L and was 1.48 time more than the normal value (Fig. 6B). After FLC transplantation (group 4) this index was returned to values similar to those in normal, untreated animals (group 1).

The Effect of Intrasplenic FLC Transplantation on Cytochrome P450 Content and Monooxygenase Activities in Liver Homogenate

In liver homogenates of normal untreated rats (group 1), CYP content was 127.1 ± 3.4 nmol mg^{-1} of protein (Table 2). Sham transplantation after prolonged CCl_4 administration resulted in almost a five times decrease of these values. Intrasplenic injection of FLC (group 4) led to an enhancement (almost two times) of CYP content from this depressed state, but did not achieve values similar to those seen in untreated animals.

Sham transplanted animals with CCl_4-induced cirrhosis (group 3) showed a reduced level of liver microsomal CYP-catalyzed oxidations. Aminopyrine-*N*-demethylation and aniline hydroxylation decreased by 17 and 4 times, respectively, compared to values seen in normal untreated rats (Table 2). The partial restoration of CYP content after FLC transplantation was accompanied by a parallel restoration of aminopyrine-*N*-demethylation and aniline hydroxylation activities in group 4 animals. This restoration of activity was more evident in aminopyrine-N-demethylation. After FLC transplantation, the activity increased by 2.2 times compared to nontransplanted CCl_4 exposed group, but did not return to normal (group 1) activities. This was also true for aniline hydroxylation activity. Sham transplantation did not result in a similar restoration of activities. FLC transplantation increased the aniline hydroxylation activity by 1.7 times compared to that seen in the sham-transplanted cirrhotic group (group 3), but it remained depressed by about 60% compared with that in normal, untreated animals (Table 2).

The Effect of Intrasplenic FLC Transplantation on Mitochondrial Respiration in Liver Homogenates

As is shown in Figure 7, the respiratory control index (RCI), which was measured as ratio V_{DNP}/V_{succ}, in liver homogenates obtained from untreated normal animals (group 1) was 4.5 ± 0.5. This testifies to a high level of coupling of oxidative phosphorylation by mitochondria in homogenates prepared from normal livers. In sham-transplanted cirrhotic animals (group 3), RCI declined by about 40% to 2.8 ± 0.2. Intrasplenic FLC transplantation (group 4) restored RCI values in liver homogenates to those seen in normal animals.

Succinate oxidation rate (V_{succ}) did not change between any of the experimental groups (Fig. 7). Oxygen consumption rate in the presence of succinate plus DNP (V_{DNF}) decreased in sham-transplanted rats with experimental cirrhosis (group 3). In animals that were treated with FLC after CCl_4 administration, the succinate plus DNP respiration rate was restored to the level of the untreated (group 1) animals (Fig. 7).

DISCUSSION

The results of the study demonstrate that a 3-month period of administration of CCl_4 at a dose of 0.2 ml/100 g twice a week resulted in morphological and biochemical changes in livers, which are typical for experimental cirrhosis, and similar to that seen by other workers under similar conditions (3,23). Cessation of CCl_4 administration did not, by itself, prevent the ongoing process of liver insufficiency and animal mortality. Indeed, the surviving sham-transplanted animals exposed to CCl_4 maintained the cirrhotic pattern of liver damage for at least 24 days (initial 10-day period after stopping CCl_4 administration followed by another 14 days after sham transplantation by intrasplenic injection). These data demonstrate that any recovery processes in organs affected by cirrhosis after cessation of CCl_4 either do not develop at all or develop only slowly, and novel therapies are needed for their stimulation.

Intrasplenic FLC transplantation was established with the aim of supporting liver function and repair. Trans-

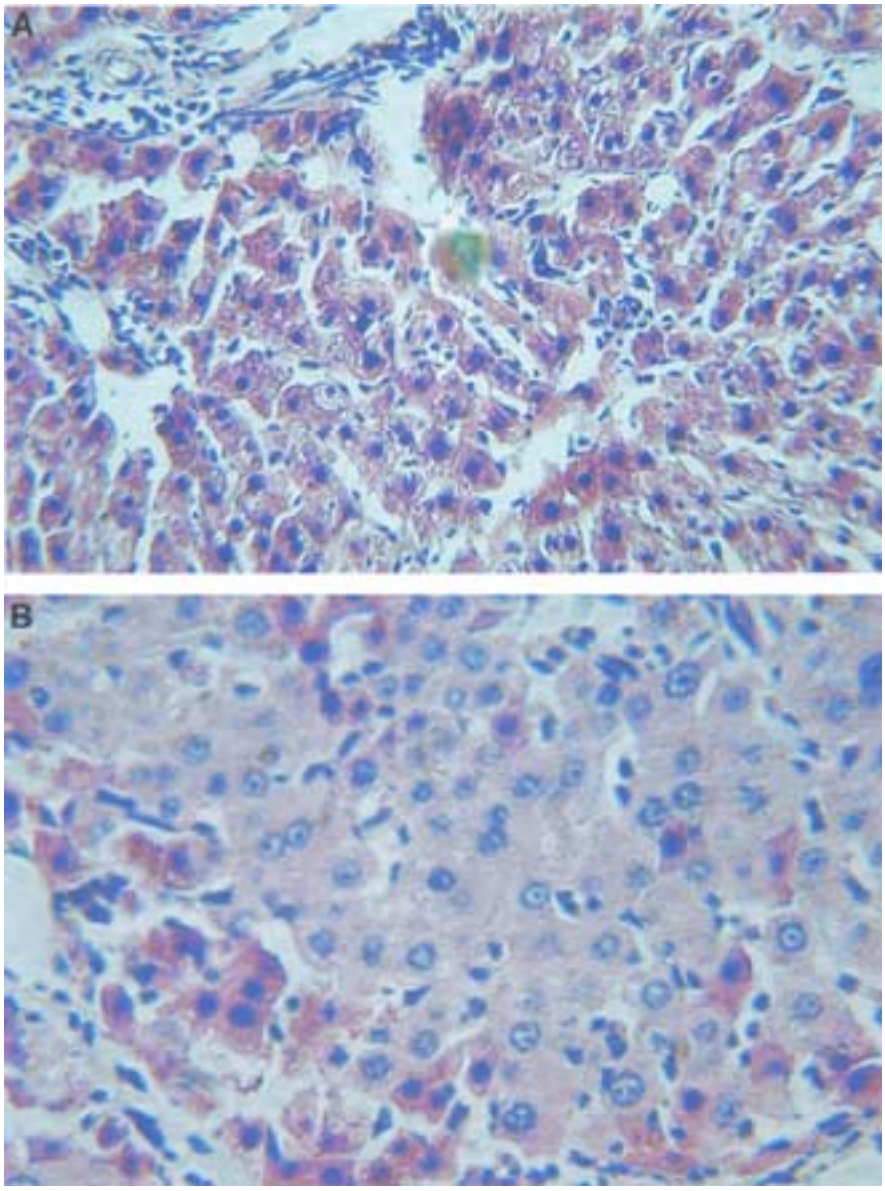

Figure 4. Histological sections of rat liver with CCl_4-induced cirrhosis 2 weeks after the sham transplantation (group 3) (A), and in the CCl_4 group with FLC transplantation (group 4) (B). Staining with hematoxyline-eosine. (A) Normal lobular architecture is disrupted; fibrosis and inflammation are still evident (original magnification 250×). (B) The parenchymal areas formed from immature or regenerating hepatocytes with light cytoplasm and enlarged nuclei (original magnification 400×).

plantation of mature hepatocytes has a long history of investigation as a method of correcting hepatic insufficiency (8,18). In animal studies, it has been established that hepatocytes injected into the splenic pulp can pass via the portal circulation into the liver (although not all cells necessarily follow this route and some remain and can survive in the spleen for prolonged periods) (40). Most investigations have been performed with isolated adult hepatocytes. However, our current study is different in that FLC were used as the transplant, and also that a xenograft model was employed. Two weeks after intrasplenic FLC transplantation, morphological evidence of activation of recovery processes in the livers of animals with CCl_4-induced cirrhosis was revealed. More-

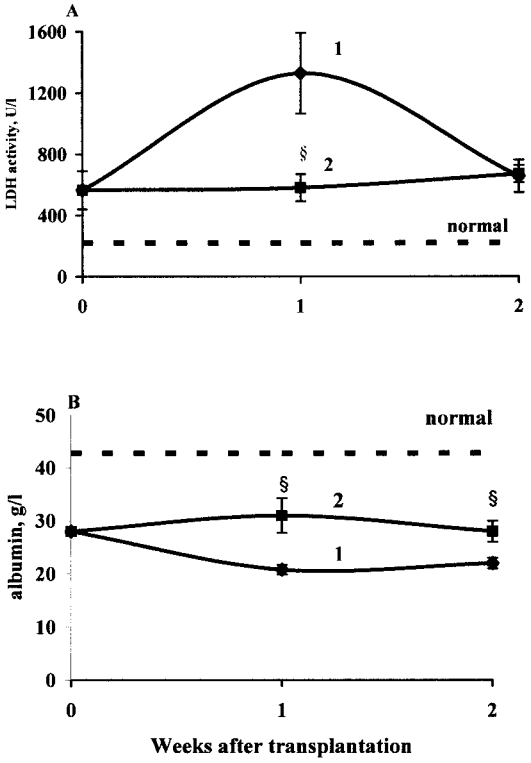

Figure 5. Serum LDH activity (A) and albumin content (B) in cirrhotic rats after sham transplantation (1) and in the cirrhotic animals after FLC transplantation (2). §$p < 0.05$ versus CCl_4, sham-transplanted group.

over, immature or regenerating cells with morphological patterns of hepatocytes (assembled in groups) were found in the livers of cirrhotic rats that received the FLC transplantation. Appearance of visible repair foci in the rat livers was accompanied by the considerable recovery of liver-specific metabolic functions. This was manifested by the decrease of TB levels and the increase of Alb content in circulating serum, as well as improvements in the detoxification and energetic functions in the liver. These combined results point to a regeneration process, with accompanying depression of free radical processes, characteristic of this liver pathology (5,7).

The restoration of RCI, which expresses the coupling of oxidative phosphorylation in mitochondria, was mainly due to stimulation of respiration rate in the state of addition of substrate and uncoupler. This measurement in liver homogenates is an indication of the total content of the enzyme complexes of the mitochondrial electron transport chain (10). In this context, it is tempting to speculate that the result may be explained by biogenesis of mitochondria in new hepatocytes, but this must await further investigation. However, taken together with information on the levels of CYP after FLC (see the following discussion), a regenerative restoration of liver functional mass is a possible explanation.

On the topic of CYP levels, our data demonstrate decreased liver total CYP content in CCl_4-induced hepatic cirrhosis, which might be expected, as it is well documented in other studies where a decrease in both CYP content and activity have been shown in animals and in patients with cirrhosis (38). In rats with experimental cirrhosis and sham transplantation, we demonstrated a sharp reduction in the activity of aminopyrine demethylation that is mostly catalyzed by CYP2CII in male rats, but also by other CYP isoforms (IAI, 2A2, 2B, and 201) (12). CYPIA is the most widely studied isoform in cirrhosis. In rats with experimental cirrhosis (either biliary cirrhosis following bile duct ligation or CCl_4-induced cirrhosis) there is a major reduction in the in vivo metabolism of caffeine, a specific substrate for this isoform (2). The inhibition of aminopyrine demethylation in rats with experimental cirrhosis indicates a reduction in the activity of CYP2C. The CYP2E1 isoform, which is responsible for aniline hydroxylation, appears to be less affected than the isoforms participating in demethylation of aminopyrine (CYP1A and CYP2C) in cirrhosis. Animal data obtained in rats with experimental cirrhosis revealed no significant changes in CYP2E1 activity (2).

The mode of action of the FLC in supporting liver

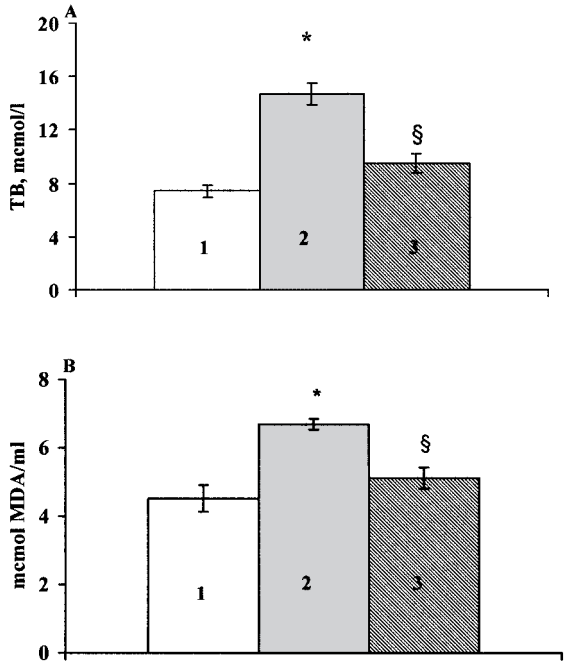

Figure 6. Serum total bilirubin level (A) and TBARS content (B) (shown as MDA levels). 1: normal (group 1) animals; 2: cirrhotic, sham-transplanted animals; 3: cirrhotic animals during 14 days after FLC transplantation. *$p < 0.05$ versus normal (group 1); §$p < 0.05$ versus CCl_4, sham-transplanted group.

Table 2. Effects of Intrasplenic FLC Transplantation on Cytochrome P450 Content and Monooxygenase Activities in Liver Homogenate of CCl_4-Induced Cirrhotic Rats ($n = 5$)

Indexes	Experimental Groups		
	Normal, Untreated	CCl_4 Exposed, Sham Transplanted	CCl_4 Exposed, FLC Transplanted
CYP (nmol mg^{-1} of protein)	127.1 ± 3.4	27.6 ± 2.3	50.5 ± 7.6*
Aminopyrine-*N*-demethylation (nmol of formaldehyde per min mg^{-1} of protein)	0.382 ± 0.002	0.022 ± 0.004	0.048 ± 0.006*
Aniline hydroxylation (nmol of *p*-aminophenol per min mg^{-1} of protein)	13.05 ± 0.25	3.11 ± 2.23	5.24 ± 0.68*

*$p < 0.05$ versus both normal (group 1) and CCl_4-exposed, sham-transplanted rats.

function in the cirrhotic rats is interesting and, from our current studies, it is not possible to define this exactly. Fetal liver of this developmental stage comprises a mixed cell population, including hepatoblasts, which are considered to be bipotential progenitor cells capable of differentiating into either mature hepatocytes or biliary epithelium, depending on the anatomical microenvironment (32). After transplantation into immune-compromised (SCID) mice, the hepatoblast population of human FLC was shown to be capable of differentiating into mature hepatocytes (20). It is also known that human cord blood contains progenitor cells capable of differentiating into mature hepatocytes in mice (again, NOD/SCID mice), in a similar model of CCl_4-induced hepatic insufficiency (25) to that which we have employed. The presence of the liver insufficiency was suggested to be a positive signal driving the engraftment and differentiation. Other workers have shown that in a similar model of CCl_4 cirrhosis, adult hepatocyte transplants are positively stimulated to engraft and replicate more in comparison to transplants performed in noncirrhotic animals (9). Immortalized adult rat hepatocytes have also been shown to support liver function in CCl_4-induced liver insufficiency (3). Elegant studies have confirmed that mature hepatocytes derived from such bone marrow progenitors express a variety of liver-specific genes (1). We have similar positive signals in our model of liver insufficiency in the rat, but this remains a xenograft situation for the human FLC, which would be expected to elicit a classical transplant rejection response. However, the immunogenicity of progenitor cells at various stages of commitment remains unclear, and in some situations, fetal cells have been considered to be less immunogenic. Indeed, in another xenograft model of transplantation of human progenitor cells into normal immune-competent rats (in this case, of human neural stem cells into adult Sprague-Dawley rats), no evidence of rejection was seen for periods up to 56 days of observation (4). In addition,

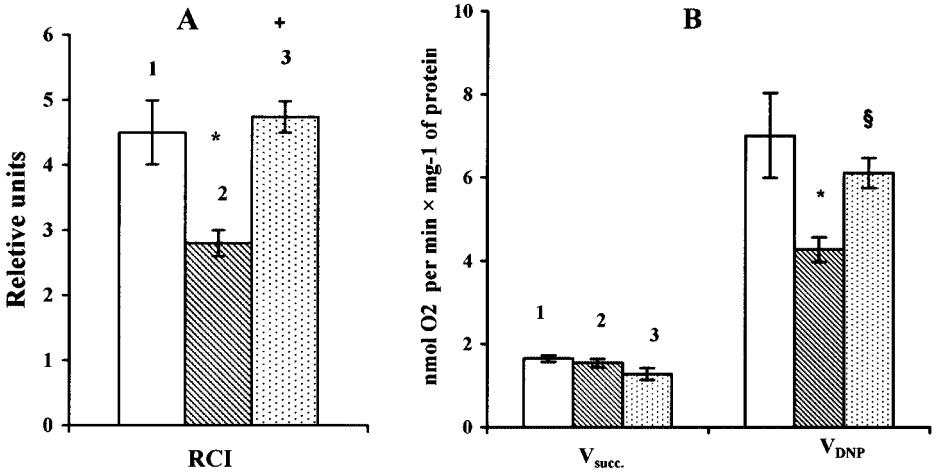

Figure 7. Respiratory control index (RCI) (A) and respiratory rates in the presence of succinate (V_{succ}) and succinate + DNP (V_{DNP}) (B) in rat liver homogenates. 1: normal (group 1) animals; 2: cirrhotic, sham-transplanted group; 3: cirrhotic animals with FLC transplantation. *$p < 0.05$ versus normal (group 1); §$p < 0.05$ versus CCl_4, sham-transplanted group.

the facts that we were transplanting a mixed population of progenitors in the FLC, and also that these were cryopreserved (see below), may have had an impact on the xenograft response. Defining the fate of human FLC in the xenograft situation will require much additional work, and this will be part of our ongoing studies. The only comments that we can offer at this stage are that the measurements of changes in mitochondrial status following FLC grafting point more towards a general hepatic repair of the animals' own livers, rather than direct support by a small number of grafted human cells. This is because mitochondrial isolation is performed by homogenization of the whole organ (allowing for small biopsies taken for other reasons), and thus the contribution to mitochondrial respiration from cells of rat origin will far outweigh those from human origin. However, it may also be that to stimulate host liver repair, the FLC may need to be viable and capable of secreting growth factors or similar agents, so the question of cell survival after FLC must remain open until further studies are specifically designed to address this.

As pointed out above, in all of the studies described here these positive results were obtained using FLC, which had been recovered from cryopreservation. Cryopreservation is in fact a routine part of the protocol for harvesting and testing of FLC within the Institute for Problems of Cryobiology and Cryomedicine. Thus, from our current observations, the applied cryopreservation protocol appears capable of yielding functional FLC, which is an observation of importance when considering banking of progenitor cells in the future for possible stem cell therapies. Cryopreservation procedures have occasionally been considered to modulate the immunogenicity of cells (30), and while it has come to be realized that this is not a generalized effect, it is possible that the exposure to cryoprotectant agents and low temperatures in our present study may have contributed to the success of the xenografted FLC. This also will need further investigation. FLC have been cryopreserved in previous work where the interest was in support of the hematopoietic cell population known to reside within fetal liver (6). Obviously, FLC are not totipotent stem cells, but nevertheless, if these cells (or cells of an earlier stem cell lineage) can be successfully cryopreserved and banked at low temperatures while still maintaining the ability to differentiate into a spectrum of mature cell types, this may be a logistically helpful procedure towards future development of cell-directed therapy for liver repair.

In summary, intrasplenic FLC transplantation in rats with CCl_4-induced cirrhosis resulted in significant restoration of a wide range of morphological, metabolic, and biochemical functions indicative of liver repair or regeneration. The major novel finding is that these effects followed from xenografting of human FLC into normal, immune-competent animals. If these findings can be substantiated and fully developed, it may suggest that progenitor cells from a wide range of sources could be considered as a therapy for liver insufficiency.

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