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HEPATOLOGY

Functional hepatic recovery after xenotransplantation of cryopreserved fetal liver cells or soluble cell-factor administration in a cirrhotic rat model: Are viable cells necessary?

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Abstract

Background and Aim: Chronic liver failure results in the decrease of the number of functioning hepatocytes. It dictates the necessity of using exogenous viable cells or/and agents that can stimulate hepatic regenerative processes. Fetal liver contains both hepatic and hematopoietic stem cells with high proliferative potential, which may replace damaged cells. Also, immature cells produce fetal-specific factors which may support the injured liver. Our aim was to test the ability of human fetal liver cells and cell-free fetal-specific factors of non-hepatic origin to stimulate recovery processes in an experimental model of carbon tetrachloride–induced cirrhosis in rats.

Methods: Cirrhotic rats were intrasplenically injected with fetal liver cells (1×10^7 cells/0.3 mL medium) or cell-free fetal-specific factors (0.3 mL/1 mg protein). Control groups received medium alone. Serum indexes, hepatic functions, and morphology were evaluated for 15 days.

Result: Human fetal liver cell transplantation was shown to abrogate the mortality of cirrhotic animals, to improve serum markers, and to restore liver mitochondrial function and detoxification. Morphological patterns of liver recovery were observed by histology. In comparison, an injection of fetal-specific factors produced similar functional recovery, whilst a more limited liver regeneration was observed by histology.

Conclusions: The positive effects of fetal liver cell and cell-free fetal-specific factors in experimental cirrhosis may result from the presence of stage-specific factors activating hepatocellular repair.

Introduction

Chronic liver failure results in a decrease in number of functioning hepatocytes. It dictates the necessity of using a source of exogenous viable cells or/and agents that can stimulate regenerative processes. Hepatocyte transplantation is an effective method for correcting of such metabolic defects. Nevertheless, the clinical application of this procedure is rare[1] because of the limited source of donor hepatocytes for isolation and concern about their long-term function after transplantation as allografts. Another promising possibility within the field of cell transplantation, which has not been explored clinically despite theoretical advantages, is fetal liver cell (FLC) transplantation. FLCs are less immunogenic than adult cells and have a high proliferative capacity. Recently we showed that human FLCs had a positive effect on cirrhotic livers and stimulated various recovery processes in the injured organ in the short term after transplantation.[2] The positive effect may result from an organ-specific functional substitution by the transplanted cells, because fetal liver during the first trimester of gestation contains both hepatic[3,4] and hemopoietic[5] stem and progenitor cells. These somatic stem cell types may form hepatocytes *in vivo* and *in vitro*.[6,7] Some groups have claimed that bone marrow–derived stem cells can be a source for developing hepatocytes after transplantation in various models of liver insufficiency,[6,8] although this remains controversial at present. Human hemopoietic stem cells have also been shown to graft and to differentiate into hepatocyte-like cells in injured mouse livers.[9]

Besides providing cell replacement, the fetal liver cells contain and produce fetal specific factors (FSF) such as cytokines and growth factors,[10–12] which have antifibrinogenic and anti-inflammatory effects in the injured liver and are able to stimulate hepatocyte proliferation.[13,14] For example, it has been shown

previously that hepatic stimulatory substance (HSS) can substantially enhance proliferative activity of the intrasplenically transplanted adult hepatocytes in a rat model of carbon tetrachloride–induced (CCl_4-induced) cirrhosis.[15] However, the ability of fetal stage-specific growth factors of non-hepatic origin to support the chronic failing liver in CCl_4-induced cirrhosis has not been studied yet. In particular, the separate effects of fetal cells and cell-free fetal-specific factors have not been investigated in the same model of liver failure.

The aim of this study was therefore to compare the ability of human fetal liver cells (FLC) and cell-free fetal-specific factors (FSF) to stimulate recovery processes in an experimental model of CCl_4-induced cirrhosis in rats. This is an important question to answer because live cell transplantation requires a different surgical and logistical approach to site-directed delivery of growth factors (the latter, in many ways more closely resembling general drug delivery).

Methods

Male white 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 termination were performed under anesthesia induced by ether inhalation.

Induction of cirrhosis

Cirrhosis was induced by intraperitoneal 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. 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 untreated animals.

For this study, there were five experimental groups:

1. Normal, age-matched untreated animals ($n = 8$).
2. CCl_4-exposed, sham-transplanted rats with CCl_4-induced cirrhosis. These rats received an 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, to permit clearance of any residual CCl_4 from the body and to avoid anesthetic complications during the surgery.
3. CCl_4-exposed, FLC transplanted rats with CCl_4-induced cirrhosis. These rats received an intrasplenic transplantation of 10^7 of cryopreserved human fetal liver cells in 0.3 mL medium ($n = 11$) 10 days after cessation of CCl_4 administration.
4. CCl_4-exposed, FSF injected rats with CCl_4-induced cirrhosis. These rats received an intrasplenic injection of 0.3 mL FSF (approximately 1 mg protein, $n = 5$), 10 days after cessation of CCl_4 administration.
5. Rats with confirmed cirrhosis ($n = 5$). These animals were studied 10 days after cessation of CCl_4 administration for the previous 3 months by removal of liver and blood samples under terminal anesthesia.

Preparation of fetal liver cell suspension

FLCs were isolated from 8- to 12-week-old human fetuses. 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 of a single cell suspension in RPMI-1640 by vibration as we described in earlier reports[16,17] and passed through a standard blood transfusion filter system. Cells were slowly mixed with an equal volume of cryopreservation medium containing 20% dimethyl sulfoxide and divided in aliquots of 1 mL in cryo-tubes and after 20 min of equilibration on ice. 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.

FSF preparation

Cell-free FSF was prepared from the remaining tissues (after liver removal) by homogenization and centrifugation ($8000 \times g$ for 40 min). The supernatant was centrifuged at $105\,000 \times g$ for 90 min. This was postmicrosomal supernatant, designated as fetal-specific cytosolyc fraction, and it was stored in liquid nitrogen until use.

Immediately before use, FLC and FSF were thawed by rapid warming in a water bath at 37°C. FLC suspensions with a viability not less than 60–62% according to a trypan blue dye test were used for the transplantation studies.

Intrasplenic injections and cell transplantation procedures

Sham-transplanted cirrhotic animals (group 2), cirrhotic animals receiving FLC transplants (group 3), and FSF-injected animals (group 4) underwent this procedure. Under anesthesia, a small surgical incision was made in the flank of the animal, and the spleens were exposed. For group 2, the animals received an intrasplenic injection of 0.3 mL of medium alone. For group 3, 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, with the incision repaired by suture. For group 4, rats received 0.3 mL of FSF, containing approximately 1 mg of protein, in the same way.

The outcome of the various treatments was followed for 2 weeks. On day 14 after intrasplenic injections, all the animals in groups 2, 3, and 4 were killed by decapitation under anesthesia.

Blood was collected for serum determinations of albumin content (Sigma, St. Louis, MO, USA), thiobarbituric acid-reactive substances (TBARS, for monitoring lipid peroxidation), and total bilirubin content (Vector-Best, Novosibirsk, Russia) which were measured by spectrophotometric means (Cary 50; Varian Inc., Mulgrave, Australia).

At the end of the study period, livers were removed from animals in all groups. Blood was washed out by venous infusion with Hank's solution, cleaned from adherent tissues, and put into cold homogenization medium containing 0.3 mol sucrose,

10 mmol Tris-HCl, and 0.5 mmol 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 vol of the medium in a Teflon Daunce glass-handle homogenizer.

Liver mitochondrial and microsomal functions were studied in the homogenates produced, as described below.

Mitochondrial and microsomal functions

Both mitochondrial and microsomal functions were studied in samples from the homogenate for groups 1, 2, 3, and 4. The respiratory activities were determined by a standard polarographic method using a Clark-type electrode in a 1 mL cell at 26°C (Rank model 20; Rank Brothers Ltd, Cambridge, UK) in a medium containing 0.25 mol sucrose, 10 mmol KH_2PO_4, 30 mmol Tris-HCl, and 0.5 mmol EDTA, pH 7.4. A solution of 8 mmol succinate was used as a substrate. At 3–4 min after the onset of substrate oxidation, 50 μM 2,4-dinitrophenol (DNP) was added to the cell. Respiratory rates in the presence of succinate and DNP were indicated as V_4 and V_{3u}, respectively. Respiratory control index (RCI) was calculated as the ratio of substrate-linked oxygen consumption rates after and prior to the addition of DNP.

Cytochrome P-450 (CYP) in liver homogenate was determined by the spectrophotometric method[18] using the coefficient of molar extinction 10.4×10^3 mol^{-1} sm^{-1} on a spectrophotometer (Specord UV VIS; Analytik Jena AG, Jena, Germany). Microsomal N-demethylase enzyme activity was estimated by formaldehyde formation.[19] The molar extinction coefficient was 8.0 $mmol^{-1}$ sm^{-1}.[20] Aniline hydroxylation was determined by measuring the formation of *p*-aminophenol.[21] The incubation medium contained 67 mmol Tris-HCl, pH 7.8; 16 mmol $MgCl_2$; and 3 mmol NADPH. Concentrations of substrates used in the assays were 8 mmol aminopirine and 3 mmol aniline. Protein concentration was determined by the method devised by Lowry *et al*.[22]

Morphology

For histology, liver specimens were fixed in neutral 10% buffered formalin, embedded in paraffin, and stained with hematoxylin–eosin.

Statistical analysis

Values are expressed as mean ± SEM. Comparisons were made using ANOVA, and the Mann–Whitney test (for non-parametric values) or Student's *t*-test were used as appropriate. Results of $P < 0.05$ were considered statistically significant.

Results

Experimental cirrhosis

During the formation of the experimental liver cirrhosis, there was a mortality of about 40% from the starting number of animals, which is typical for this model.[23] When the CCl_4 injection was stopped after 3 months, there was an ongoing mortality with reduction in motional activity and decline in body weight observed; furthermore, changes in hair colour were noted and there was partial hair loss. Most of the cirrhotic animals had dyspepsia and tendency to develop skin disease and abscesses. These symptoms testified to the serious metabolic derangements in the experimental animals even after cessation of CCl_4 administration.

Table 1 Blood serum indices in cirrhotic rats 2 weeks after intrasplenic injections of fetal liver cells (FLC) or fetal-specific factors (FSF) ($n = 5–7$)

Experimental groups	Biochemical indices in blood serum		
	Total bilirubin (μmol/L)	Albumin (g/L)	TBARS (μmol MDA/L)
Normal, untreated	7.4 ± 0.5	42.8 ± 2.3	4.5 ± 0.39
Confirmed CCl_4-induced cirrhosis	17.6 ± 0.9*	28 ± 1.2*	9.6 ± 0.7*
CCl_4-exposed, sham-transplanted	14.7 ± 0.9*	22.2 ± 1.1*	6.7 ± 0.16*
CCl_4-exposed, FLC transplanted	9.5 ± 0.7*,**	27.7 ± 1.9*,**	5.1 ± 0.31**
CCl_4-exposed, FSF injected	8.3 ± 0.6**	29.3 ± 0.25*,**	5.2 ± 0.5**

*$P < 0.05$ *vs* normal; **$P < 0.05$ *vs* sham-transplanted group.
Values are also shown for animals with confirmed cirrhosis for comparison.
CCl_4, carbon tetrachloride; TBARS, thiobarbituric acid-reactive substances.

The morphological confirmation of the extent of liver damage was carried out 10 days after the cessation of the CCl_4 injections (group 5, confirmed cirrhosis). The maturation of intrahepatic connective tissue occurred and acute inflammation abated in the affected organs at that time. We observed marked changes in the organ architecture which were manifest as disorders in the trabecular structure of the liver lobules and fibrosis (these have been described previously[2]). Hepatocytes located in central perivenous zones showed evidence of necrosis. Considerable lymphoid-histiocytic cell infiltration was observed in the zones of fibrosis, around the portal tracts and in the liver parenchyma.

The total bilirubin concentration in the serum of animals in group 5 at day 10 after the cessation of the CCl_4 injections was 2.3 times higher than the value in normal untreated rats, while serum albumin concentrations were 1.5 times lower (refer to Table 1). 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.

The effect of FLC and FSF administration on animal survival rate and liver morphology

The experimental animals in groups 2 (sham transplanted), 3 (FLC transplanted), and 4 (FSF injected) were under daily observation for up to 14 days after receiving intrasplenic injections. The recovery period was most complicated in group 2, the sham-transplanted group; five animals died during first week after the operation, reducing the survival rate to 58% ($P < 0.05$ *vs* age-matched controls). In group 3, the FLC transplantation group, only

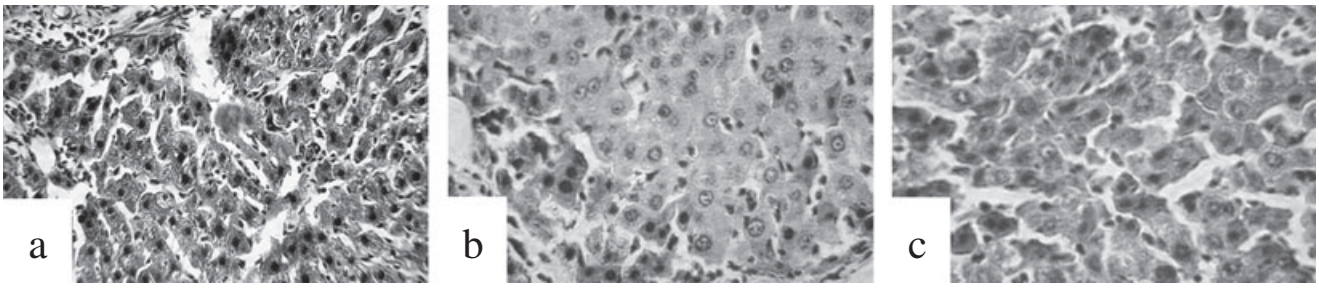

Figure 1 Histological sections of rat liver with CCl_4-induced cirrhosis in group 2, the sham-transplantation group (a), and in group 3, the CCl_4 group with fetal liver cell (FLC) transplantation (b), and group 4, which was given fetal-specific factors (FSF) injections (c). Staining with hematoxylin–eosin. Normal lobular architecture is disrupted, developing fibrosis can be seen throughout the liver lobule, inflammatory infiltrates are present, and many hepatocytes exhibit a shrunken morphology with dark, pycnotic nuclei; magnification ×250 (a). Parenchymal areas formed from immature or regenerating hepatocytes with light cytoplasm and enlarged nuclei can be seen, randomly distributed throughout the liver tissue. Some shrunken hepatocytes are still evident and disruption of lobular architecture has not been fully reversed; magnification ×400 (b). Immature or regenerating hepatocytes spread diffusely through the parenchyma and coexisting as individual cells alongside hepatocytes with shrunken and pycnotic cells; magnification ×400 (c).

one animal died during the 14 days, and survival rate was 91%. In group 4, the FSF injection group, no animals died.

Investigation of histological sections of cirrhotic livers in group 2, the sham-transplanted group, showed no noticeable changes in the organs after 14 days compared to group 5, the confirmed cirrhosis group. The false segmentation of the liver parenchyma, with hypertrophy of fibrous connective tissue, disruption of the plates of hepatocytes, and massive lymphoid infiltration of the parenchyma was still clearly evident. (Fig. 1a). The hepatocytes manifested signs of polymorphism, with some showing normal histological features, while others expressed granularity in cytoplasm and cytoplasmic vacuolization. Cell with nuclei at different stages of necrosis and degeneration were seen, alongside cells with normal cellular nuclei. Occasional binucleate hepatocytes were sometimes found.

In contrast, investigation of histological liver sections from group 3 animals, those which had received FLC transplantation, showed morphological changes which 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 in some areas of hepatocytes when compared to livers from group 2 (sham-transplanted) cirrhotic animals (Fig. 1b). Some false segmentation of the liver parenchyma remained, but hepatocytes with normal morphological features were found more frequently throughout the lobules. There were typical liver parenchymal areas formed from such hepatocytes, with light cytoplasm and large nuclei containing chromatin and nucleoli (typical of immature or regenerating hepatocytes), but full recovery of normal lobular structure was still not evident. Binucleate hepatocytes were found more often than in the sham-transplanted group samples.

To estimate the contribution of intact cells to recovery processes under CCl_4-induced cirrhosis, we injected cell-free FSF from fetal tissues of non-hepatic origin. Investigation of histological sections of rat livers 2 weeks after FSF administration showed that together with destructive and dystrophic changes typically seen in cirrhotic livers, there were morphological symptoms of activation of recovery processes in the organ (Fig. 1c). However, the characteristics of these changes were different in the FLC and FSF groups. Immature cells with morphological features of hepatocytes were also found in the histological sections after FSF treatment, but they did not form large assemblies and were spread diffusely throughout the parenchyma as single cells or groups of cells in small numbers.

Effect of FLC and FSF administration on serum indices of hepatic function

Two weeks after the surgical procedure, the albumin content in the sham-transplanted cirrhotic group was 1.9 times lower and the total bilirubin level was 2 times higher than in the untreated, normal animals (group 1) (Table 1). In contrast, FLC transplantation (group 3) resulted in an increase of albumin content to 27.7 ± 1.9 g/L and a decrease in total bilirubin level to 9.5 ± 0.7 μmol/L.

Two weeks after the operation, the TBARS in blood serum in the sham-transplanted cirrhotic group (group 2) reached 6.7 ± 0.16 μm/L and this was 1.48 times higher than the level found in normal controls. After FLC transplantation (group 3), this values were similar to those in normal, untreated animals (group 1).

FSF administration (group 4) also had a positive influence on biochemical profiles of blood serum, as did FLC (Table 1).

The effect of intrasplenic FLC and FSF injection on CYP P-450 content and CYP monooxygenase activities in liver homogenate

In liver homogenates of normal untreated rats (group 1), CYP content was 127.1 ± 3.4 nmol $\times$ mg^{-1} of protein (Fig. 2a). Animals with sham transplantation after prolonged CCl_4 administration had a CYP content almost five times lower. Intrasplenic injection of FLC (group 3) and FSF (group 4) led to an enhancement, almost by two times, of the CYP content from this depressed state, but did not completely return to the values seen in untreated animals.

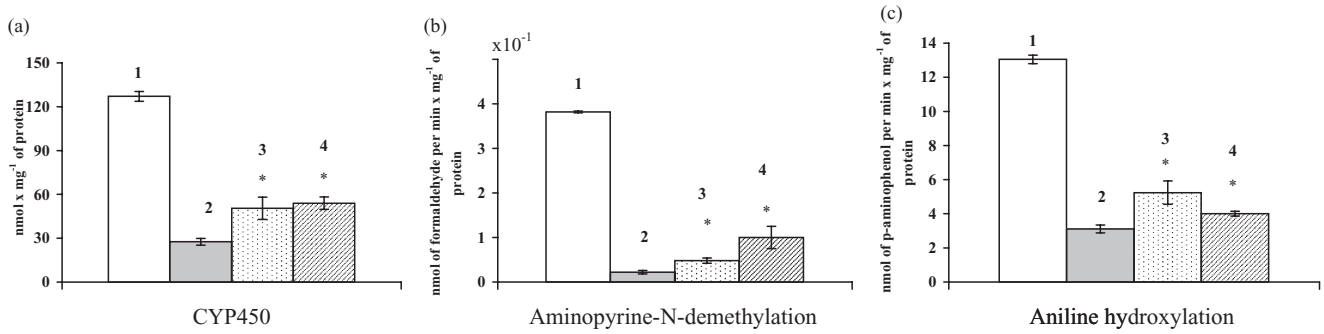

Figure 2 Cytochrome P-450 (CYP) content (a) and monooxygenase activities (b,c) in liver homogenate of normal untreated rats (1); CCl_4-induced cirrhotic rat in the sham-transplanted control group (2); and after injections of fetal liver cell (FLC) (3) and fetal specific factors (FSF) (4). $P < 0.05$, *vs* the normal and sham-transplanted groups.

Table 2 Effects of intrasplenic fetal liver cell (FLC) and fetal-specific factor (FSF) injections on respiratory rate and respiratory control index (RCI) in liver homogenates of the CCl_4-induced cirrhotic rats ($n = 5$)

Experimental groups	Indexes		
	V_{3u} (nmol O_2 per min × mg^{-1} of protein)	V_4 (nmol O_2 per min × mg^{-1} of protein)	RCI (V_{3u}/V_4)
Normal, untreated	7.3 ± 1.07	1.65 ± 0.08	4.5 ± 0.49
CCl_4-exposed, sham-transplanted	4.17 ± 0.3*	1.54 ± 0.1	2.8 ± 0.2*
CCl_4-exposed, FLC transplanted	6.11 ± 0.36**	1.28 ± 0.15	4.74 ± 0.24**
CCl_4-exposed, FSF injected	5.28 ± 0.67	1.37 ± 0.13	3.8 ± 0.38

*$P < 0.05$ *vs* normal; **$P < 0.05$ *vs* sham-transplanted group.
Values are also shown for sham-transplanted animals for comparison.
CCl_4, carbon tetrachloride; V_{3u}, uncoupler-stimulated respiratory rate; V_4, succinate oxidation rate.

When looking at CYP-related enzyme activities, sham-transplanted animals with CCl_4-induced cirrhosis (group 2) showed reduced levels of liver microsomal CYP-catalyzed oxidations. Aminopyrine-*N*-demethylation and aniline hydroxylation decreased by 17 and four times, respectively, compared to values seen in normal untreated rats (Fig. 2b,c).

The partial restoration of CYP content after FLC and FSF injection was accompanied by a parallel restoration of aminopyrine-*N*-demethylation and aniline hydroxylation activities in animals in groups 3 and 4. This restoration of activity was more evident in aminopyrine-*N*-demethylation. After both FLC and FSF injection, the activity was 2.2 and 4.4 times higher, respectively, compared to the non-transplanted CCl_4 exposed group. After FSF injection, the values were more likely to be ameliorated compared to those seen after FLC transplantation ($0.05 < P < 0.1$).

This was also true for aniline hydroxylation activity. Sham transplantation did not result in any restoration of enzyme activities. FLC transplantation increased the aniline hydroxylation activity by 1.7 times compared to that seen in the sham-transplanted cirrhotic group, but it remained depressed by about 60% compared with the values seen in normal, untreated animals (Fig. 2c). Aniline hydroxylation activity also increased after FSF injection by 1.3 times. The difference in values of these experimental groups was not significant.

The effect of intrasplenic FLC and FSF injection on mitochondrial respiration in liver homogenates

As is shown in Table 2, the RCI, which was measured as ratio V_{3u}/V_4, 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 2), RCI declined by about 40% to 2.8 ± 0.2. Intrasplenic FLC transplantation (group 3) restored RCI values in liver homogenates compared to those seen in normal animals. The effect of intrasplenic FSF injection (group 4) was not so clearly expressed as after FLC transplantation. The values recovered only to an intermediate level in the untreated and sham-transplanted groups.

Succinate oxidation rate (V_4) did not change in any of the experimental groups. Oxygen consumption rate in the presence of succinate + DNP decreased in sham-transplanted rats with experimental cirrhosis (group 2). In animals which were treated with FLC after CCl_4 administration, the succinate + DNP respiration rate was restored to the level of the untreated animals (group 1). FSF injection had a lesser effect on DNP-stimulated succinate oxidation compared to FLC transplantation.

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 those seen by other workers under similar conditions.[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 for 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 livers after cessation of CCl_4 in this model of hepatic fibrosis either do not spontaneously develop at all or develop only slowly. It is known that spontaneous recovery from CCl_4-related fibrosis is possible via apoptosis of the involved inflammatory cells (hepatic stellate cells), but this generally requires several months for significant liver tissue remodelling to occur,[24,25] whereas our study period was much shorter. Also, the ongoing cirrhosis in our model in the sham-transplanted group indicates an absence of significant spontaneous recovery.

In our study, both FLCs and FSFs were assessed as agents for stimulating recovery of the liver with CCl_4-induced cirrhosis in the rat. The results for survival, liver morphology, and biochemical recovery in this model after FLC are similar to those which have been reported previously in full,[2] and are shown here for comparison with FSF treatment. These FLC cells are known to produce hepatocyte growth factor (HGF) and other liver- and development-dependent substances.[12,26,27] For the purposes of identifying the role of viable cells in promoting liver recovery due to fetal specific factors alone, another group received cell-free FSF of fetal non-hepatic tissues of mainly mesodermal origin via the same route.

Intrasplenic injection of both FLC and FSF prevented animal mortality to an equal extent and almost completely normalized TBARS content in blood serum; this confirms the role of free radical processes in lesions induced by chronic CCl_4 administration,[28,29] and indicates that living cell activity is not necessary for this process of repair against free radical damage. In fact, we have recently shown that FSF pretreatment can enhance liver antioxidant defenses in a model of hepatic cold preservation and reperfusion, supporting the evidence that a strong protective effect resides within the soluble factors in the FSF extract.[30]

Two weeks after intrasplenic injection in both the FLC and FSF groups, there were morphological signs testifying to the activation of recovery processes in the cirrhotic livers. Appearance of foci of visible repair was accompanied by the considerable recovery of liver function shown by the biochemical indices. This was manifested by the decrease in total bilirubin levels and the increase of albumin content in the blood serum, as well as the improvement in the detoxification and energetic liver enzyme functions in seen in the studies on liver microsomes and mitochondria. We cannot at present rule out the possibility that, in the FLC-transplanted group, surviving cells of donor origin were present in the regenerating nodules, and this will require further study for clarification. No immunosuppression was given in these xenografts, and thus rejection of the grafted cells would be expected to occur at some point. 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 of up to 56 days of observation.[31] Human stem cell populations have been shown to survive for prolonged periods as differentiated hepatocytes in mice with liver damage induced by CCl_4, but these animals were an immunodeficient strain of mouse.[8]

The restoration of RCI, which expresses the coupling of oxidative phosphorylation in mitochondria, was mainly due to stimulation of the respiration rate in the condition when substrate was added in the presence of an uncoupler. This measurement in liver homogenates is an indication of the total content of the mitochondrial enzyme complexes of the electron transport chain.[32] In this context, it is tempting to speculate that the result may be explained by biogenesis of mitochondria in recovering or replicating hepatocytes, but this must await further investigation. However, taken together with information on the levels of CYP after FLC and FSF (see following discussion), a regenerative restoration of liver functional mass is a possible explanation. Investigation of the energetic state of the livers showed that both FSF and FLC exercised one-way positive influences; however, the FSF effects were slightly lower than those seen with FLC.

Hepatic biotransformation capacities are reflected by the CYP levels, and 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.[33] In rats with experimental cirrhosis and sham transplantation, we demonstrated a sharp reduction compared to normal animals in the activity of aminopyrine demethylation which is mostly catalyzed by CYP 2CII in male rats, but also by other CYP isoforms (IAI, 2A2, 2B, and 201).[34] CYP IA 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 CYP isoform.[35] The inhibition of aminopyrine demethylation in rats with experimental cirrhosis indicates a reduction in the activity of CYP 2C. The CYP 2E1 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 previously in rats with experimental cirrhosis revealed no significant changes in CYP2E1 activity.[33] In our present study, intrasplenic injection of FLC and FSF in animals with CCl_4-induced cirrhosis resulted in a significant restoration of both CYP P-450 contents and their activities.

Biochemical and histological results demonstrated that fetal cell-free extracts of non-hepatic origin, as well as FLC transplantation, promoted activation of recovery processes in livers with CCl_4-induced cirrhosis. It is known that development of chronic liver pathology is accompanied by profile changes in both cytokines and growth factors. When chronic hepatitis is transformed into cirrhosis, production of pro-inflammatory agents, specifically TGF-β1, occurs, which results in the intensification of fibrosis.[36] The ratio and concentrations of inflammatory and anti-inflammatory cytokines determine the balance between the devel-

oping pathological and reparative processes.[37,38] We propose that the 'cocktail' of fetal stage-dependent growth factors changes the balance of the linked processes towards the direction of repair and regeneration. This hypothesis is supported by our previous study,[30] where FSF administration attenuated free radical damage in a liver model of cold preservation/reperfusion injury.

However, at the same time there were morphological differences in the livers of animals treated with either FSF or FLC. Immature cells with the morphological traits of hepatocytes were found assembled in groups after FLC transplantation, while after FSF injection, there were many fewer immature hepatocytes in the liver sections. These hepatocytes did not form assemblies, but rather they were spread diffusely through the whole hepatic parenchyma. There are a number of possible explanations for these differences. Firstly, in the case of FLC, migration of hepatocyte precursors transplanted into the spleen and then moved to the liver, followed by their further expansion; however, after FSF, a stimulation of the livers' own hepatic stem cell pool by FSF occurred. Secondly, cell fusion between transplanted immature liver stem cells and host liver cells was stimulated; this has been documented where cord blood–derived hemopoietic stem cells were grafted into mice with CCl_4-induced cirrhosis.[9] Thirdly, the short-term survival of the transplanted FLC acted as a 'slow-release' depot for the kinds of cytokines and growth factors present in FSF. These suggestions are controversial and will require much further work for clarification.

It is clear that FSF injection reproduced nearly all the positive effects seen after FLC transplantation. Positive effects of injection of individual growth factors, such as interleukin-10, on CCl_4-induced cirrhosis have been reported,[39] and FSF may provide an enhanced mixture of such substances. Multiple injections of IL-10 over a prolonged period were provided by the intraperitoneal route previously.[39] In the present study, only one single injection of FSF produced these positive effects on liver functional recovery, but the mixture was provided via a route (intrasplenic injection) which would lead to direct portal inflow to the liver. In addition, the ability to store the FSF in an active state by freezing it in liquid nitrogen is a small but positive step towards the development of a possible long-term bank of bioactive components. Interestingly, liver cell cytosolic fractions were shown to have potential for stimulating liver repair in damaged organs many years ago.[40] Thus, even adult hepatocytes may contain soluble factors which may be of therapeutic value. Whether these are in any way similar to those in the FSF extracts is not known, but our current studies do provide evidence to suggest the importance of soluble factors without the need for transplantation of living cells.

In summary, the positive effects of FLC and FSF in this model of cirrhosis may be due to the presence, both in FLC and FSF, of stage-specific growth factors activating hepatocellular repair. Further work is needed to elucidate the potential mechanisms of hepatic stem cell support in liver disease.

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