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Intrasplenic or Subperitoneal Hepatocyte Transplantation to Increase Survival after Surgically Induced Hepatic Failure?

G. Gäbelein[a] A.K. Nüssler[b] F. Morgott[a] Y. Ping[a] N. Nüssler[c] P. Neuhaus[a] M. Glanemann[a]

[a]Department of General, Visceral, and Transplantation Surgery, Charité, Campus Virchow Klinikum, Universitätsmedizin Berlin, Berlin, [b]Department of Traumatology, Technical University Munich, and [c]Department of General Surgery, Klinikum Neuperlach, Munich, Germany

Key Words
Liver surgery · Acute liver failure · Hepatocyte transplantation

Abstract
Background: As a basis for future clinical questions, we evaluated the efficacy of hepatocyte transplantation in a surgical model using a subperitoneal or intrasplenic approach for cell implantation. ***Methods:*** In rats, acute liver failure was induced by subtotal hepatectomy. Series of allogenic hepatocyte transplantations were performed by varying cell number, site, and sequence of cell transplantation. ***Results:*** Following subperitoneal or intrasplenic cell implantation subsequent to liver surgery, no survival benefit was achieved when compared to the control groups. However, intrasplenic cell implantation 24 h prior to liver surgery revealed a statistically significantly higher animal survival (72 vs. 29%). ***Conclusion:*** According to our experience, both timing and site of cell implantation played an important role in hepatocyte transplantation. Intrasplenic hepatocyte transplantation 1 day before liver surgery showed the best results in terms of survival. Consequently, we were able to establish a model of hepatocyte transplantation which may be the basis for further investigations evaluating potential treatment modalities to overcome deleterious postoperative liver insufficiency.

Introduction

Presently, liver transplantation is the only treatment option available for acute liver failure. However, because of donor organ shortage, mortality rates remain high [1–3]. In the last three decades, research has focused on the development of alternatives or supportive measures to treat acute liver failure. Because it is thought that the function of the liver can only be replaced with a biological substrate, hepatocyte transplantation is one of the most studied techniques, and has been proposed as an alternative to whole-organ transplantation to support many forms of hepatic insufficiency [4–6]. In addition, hepatocyte transplantation has also been proposed as a liver-directed gene therapy for a number of inherited hepatic disorders by transplanting either freshly isolated or genetically altered hepatocytes [7].

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Matthias Glanemann, MD
Department of General, Visceral and Transplantation Surgery, Charité
Campus Virchow Klinikum, Universitätsmedizin Berlin
Augustenburger Platz 1, DE–13353 Berlin (Germany)
Tel. +49 30 450 552001, Fax +49 30 450 552900, E-Mail matthias.glanemann@charite.de

Table 1. Studied groups are listed and pooled in series

Series	Site of implantation	Day of implantation	Cells ($\times 10^6$)	n	Mean BW (day 0), g	Resected liver mass, g
1	subperitoneal	0	0	9	279 ± 19	9.6 ± 1.4
	subperitoneal	0	24	8	269 ± 28	9.5 ± 0.9
2	subperitoneal	–1	0	6	286 ± 10	8.1 ± 0.4
	subperitoneal	–1	24	5	296 ± 4	7.9 ± 0.3
3	spleen	0	0	10	253 ± 20	8.3 ± 0.8
	spleen	0	24	3	232 ± 19	7.5 ± 0.2
4	spleen	–1	0	14	287 ± 13	7.7 ± 0.8
	spleen	–1	16	9	292 ± 14	9.2 ± 0.5
	spleen	–1	24	18	266 ± 22	8.3 ± 0.8
	spleen	–1	32	8	278 ± 9	8.4 ± 1.0
	spleen	–1	48	6	257 ± 19	9.2 ± 0.5

Site and day of cell implantation differed as well as dosage of injected rat hepatocytes (saline solution in control animals) following subtotal hepatectomy to induce acute liver failure.

n = Group size; BW = body weight.

Since it was widely believed that the optimal site for hepatocyte transplantation was the liver itself [8], trials were designed to transplant hepatocytes directly into the liver via the portal circulation. However, investigators have found that ectopic transplantation of hepatocytes to areas of the body other than the liver, such as under the kidney capsule, subcutaneous space and peritoneal cavity, also leads to therapeutic efficiency [9, 10]. Furthermore, ectopic transplantation of hepatocytes has some advantage over the intraportal transplantation, since a large number of hepatocytes can be transplanted to most of the ectopic sites with minimal invasive procedures.

Thus, the development of a surgical model using hepatocyte transplantation is the subject of this report. We compared the practice of intrasplenic injection with an as yet not evaluated method – the subperitoneal injection – by varying cell count and time of implantation.

Materials and Methods

Animals

In our study, we used 96 male Wistar rats (Fa. Harlan-Winkelmann, Borchen, Germany), weighing between 200 and 300 g, which were housed in our animal facility. Eleven groups were merged into 4 series as shown in table 1. Cell count and site of transplantation as well as sequence of cell implantation varied according to the protocol.

Rats were maintained with commercial standard laboratory rat chow, a 12-hour light/dark cycle, a constant temperature of 25°C and relative humidity of approximately 40%. They were acclimatized to our laboratory conditions for 1 week prior to the experiments. Before and after the intervention, rats were allowed free access to food and tap water. Postoperatively, 5% dextrose was offered ad libitum. An analgesia test was performed with tramadol administered subcutaneously adapted to body weight once after the surgical procedure. Immunosuppression was not applied.

Body weight, signs of encephalopathy, and animal survival were recorded daily and compared between the corresponding groups. Encephalopathy score based upon the coma scale described by Nagata et al. [11] was used to assess the grade of encephalopathy in the postoperative course (description of behavior: 5 points = spontaneous and interested ramble; 4 points = reserved spontaneous activity; 3 points = temporary activity after disturbing excitability; 2 points = no activity but delayed erecting after laying down in a lateral position; 1 point = no activity, weak vital signs, barely able to drink; 0 points = positive corneal reflex as single reaction). Moreover, this score was also used to decide on premature harvesting (in case of 0–1 points) due to incompatibility with survival.

At the end of the observation period, the surviving animals were exsanguinated, and blood and liver samples were stored for enzymatic, biochemical and histological analyses. Serum bilirubin, albumin, ALT, AST, and GLDH were analyzed using commercially available reaction kits (Roche Diagnostics, Mannheim, Germany) at the Institute of Laboratory Medicine, Charité, Humboldt University Berlin, Germany. After tissue removal, the liver, lung, and – depending on the implantation locus – spleen or peritoneum were fixed in formalin, cut and stained with hematoxylin and eosin (HE) for histological analysis.

All procedures were reviewed and approved by the local government (Senator für Gesundheit und Soziales, Berlin) and carried out according to the European Union regulations for animal experiments, and were thus within the guidelines for the care and use of laboratory animals.

Cell Isolation

Male Wistar rats also served as a donor of cells for transplantation. Primary rat hepatocytes were isolated by a two-step collagenase perfusion technique according to a standard procedure of our laboratory [12]. Determination of viability was done by the trypan blue exclusion test using a Neubauer chamber. Initial cell viability after cell isolation ranged between 67 and 88%. However, when a Percoll gradient technique was performed on these cells, we reached a viability >93%, determined by the trypan blue exclusion test. Then, cells were suspended in 0.7 ml of phosphate-buffered saline.

Surgical Procedure and Cell Implantation

Induction of acute liver failure was performed by subtotal liver resection according to a modified protocol of the method described by Higgins and Anderson [13]. Left and median liver lobes were removed after central ligature with a 4-0 absorbable, synthetic, braided thread. The right upper and lower lobes were rendered necrotic by ligation of the common right liver lobe pedicles using a braided silk thread. Both omental liver lobes and parts of the liver tissue surrounding the intrahepatic portion of the infe-

rior vena cava remained, together representing approximately 10% of the total liver mass. With this procedure described by Eguchi et al. [13], highly reproducible symptoms of fatal hepatic failure, including severely impaired ability of the residual liver tissue to regenerate, can be achieved.

Hepatocyte implantation was performed by slow injection of isolated rat hepatocytes suspended in 0.7 ml of phosphate-buffered saline using a 25-gauge needle connected to a 1-ml syringe. In case of subperitoneal implantation (fig. 1a), the needle was tunneled tangentially tight under the peritoneum before injection to avoid cell leakage after removing the needle. In case of intrasplenic injection (fig. 1b), blood flow in both splenic arteries and veins was clamped before injection and remained occluded for a further 5–7 min to avoid immediate passage of cells into the portal vein. The injection site was ligated to prevent cell leakage and bleeding. Instead of hepatocytes, a saline solution (0.9% sodium chloride of analogous volume) was used in each series for appropriate controls.

Statistics

Results were expressed as means ± SEM. After proving the assumption of normality and equal variance, differences between groups were assessed using ANOVA (overall differences) followed by the appropriate post hoc method. Differences in survival were measured using the log rank test. Overall statistical significance was set at $p < 0.05$. Statistics were performed using the software package SPSS (SPSS Inc., USA).

Results

Survival Study

In series 1–4, location and sequence of implantation were varied. In all control groups – loaded with saline chloride solution – approximately one third of animals survived the observation period after subtotal hepatectomy with a mean survival of 3 days, independent of implantation site or sequence (fig. 2).

Subsequent transplantation of rat hepatocytes (24×10^6) subperitoneally did not affect 5-day survival, reaching 38 versus 33% in controls (series 1). However, cell implantation of rat hepatocytes (24×10^6) into the spleen at the day of surgery revealed a significantly worse survival (0 vs. 20%, $p = 0.001$), despite the same number of transplanted cells (series 3). Within a few hours after surgery, all animals in the group treated with hepatocytes had neurological symptoms like jumpiness and cramps and died. However, hepatocyte transplantation (24×10^6 cells) into the spleen 1 day before liver resection significantly affected 5-day survival (series 4), reaching 72 versus 29% (fig. 2). Mean survival was statistically significantly improved from 3 to 5 days in this group ($p = 0.009$). However, this observation was not made in case of subperitoneal hepatocyte transplantation (24×10^6) 1 day

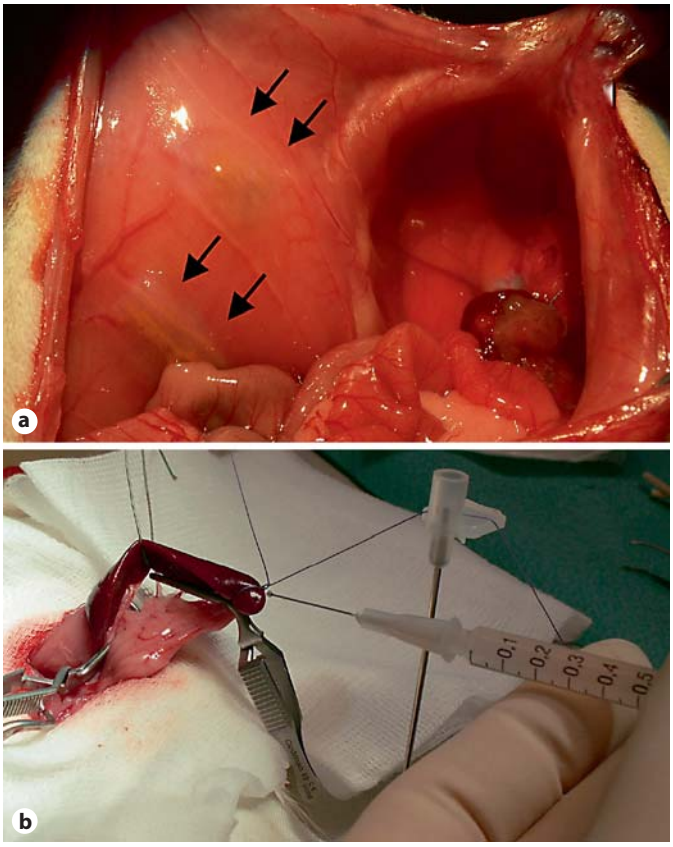

Fig. 1. Hepatocyte implantation was performed subperitoneally (**a**) or into the spleen (**b**).

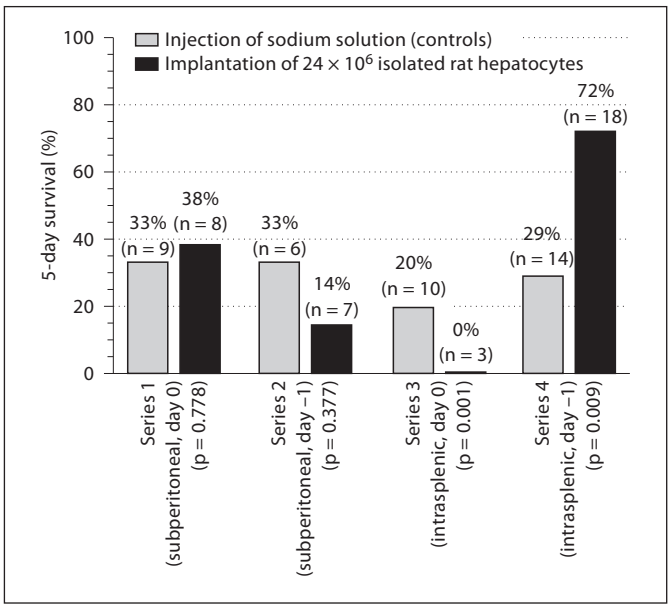

Fig. 2. Survival following subtotal hepatectomy and different sites of hepatocyte transplantation.

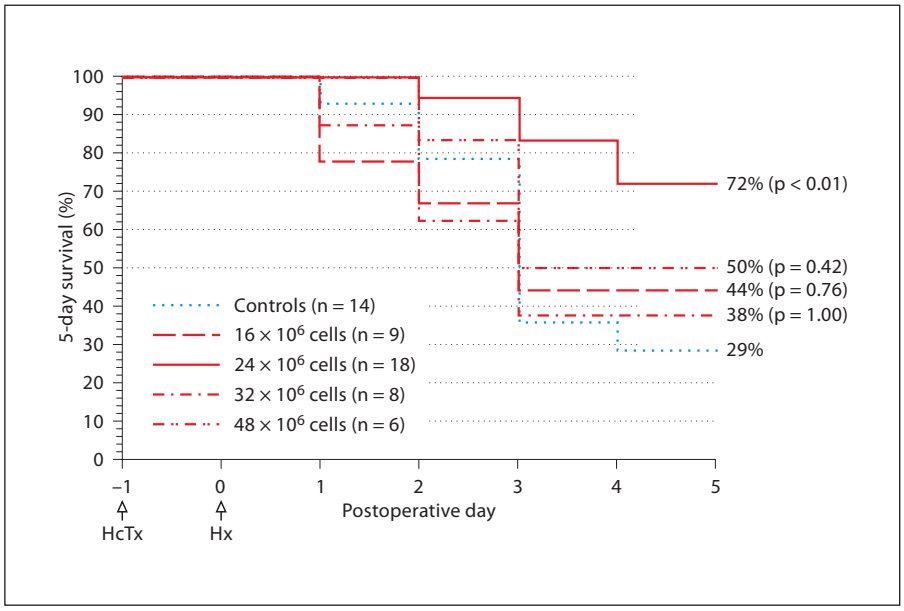

Fig. 3. Survival following subtotal hepatectomy and intrasplenic transplantation of rat hepatocytes 1 day prior to surgery (series 4) using different cell numbers. HcTx = Hepatocyte transplantation.

before surgery (series 2). In these animals, mean survival was 3 days like in the controls, and only 1 animal survived until the end of the observation period (5-day survival: 14%, p = 0.377; fig. 2).

Varying the number of transplanted cells in series 4 (intrasplenic injection 1 day prior to surgery), we observed that an increase from 24 × 10^6 to 32 × 10^6 and 48 × 10^6 transplanted hepatocytes did not automatically increase overall survival (fig. 3). Indeed, 5-day survival was similar in animals loaded with 16 × 10^6, 32 × 10^6 and 48 × 10^6 rat hepatocytes (44, 38 and 50%, respectively).

Body Weight, Encephalopathy, Blood Findings

Postoperative body weight and encephalopathy score were evaluated daily after surgery. Since all animals lost weight postoperatively compared to the preoperative level, only survivors showed an increase in body weight within the observation period. However, no statistically significant differences were observed between the different groups or series (data not shown).

A similar observation was made for severity of postoperative encephalopathy. As expected, a decrease in the encephalopathy score was noticed after liver surgery in all groups. Almost all of the survivors reached a score of 5 points on the fourth or fifth postoperative day. However, there was no statistically significant difference between the groups or series (data not shown).

In addition, laboratory findings (albumin, bilirubin, AST, ALT, GLDH) in the surviving animals did not reveal any statistically significant differences 5 days after surgery, except GLDH levels which were (partially) significantly lower in animals who had undergone intrasplenic cell injection [series 4: 16 × 10^6: 51 ± 31 U/l (p = 0.035) or 24 × 10^6: 66 ± 43 U/l (p = 0.04), but not in 32 × 10^6: 45 ± 35 U/l (p = 0.49), 48 × 10^6: 391 ± 120 U/l (p = 0.65)] 1 day prior to liver surgery when compared to controls with 391 ± 121 U/l.

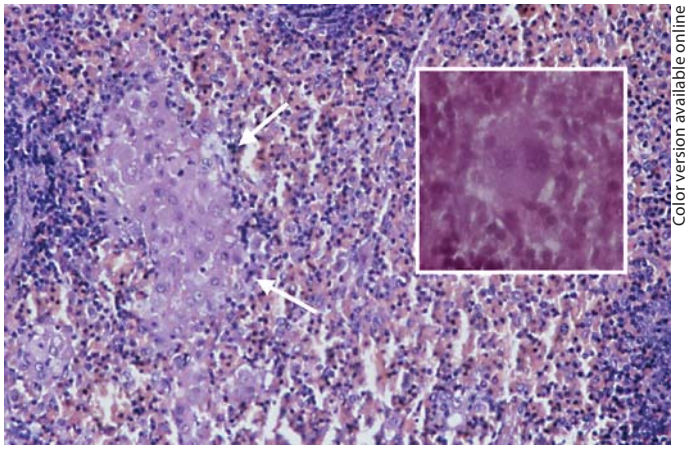

Fig. 4. Hepatocytes in the spleen 6 days after implantation of 24 × 10^6 primary rat hepatocytes into the splenic pulp, and 5 days after subtotal hepatectomy (HE staining, ×200 and ×400).

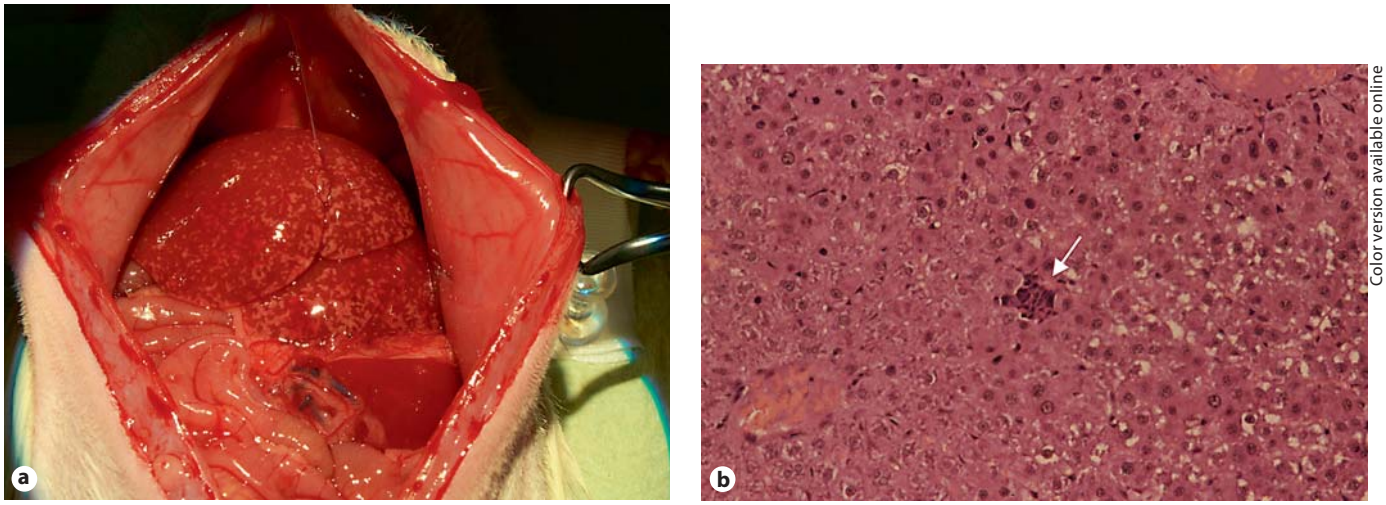

Fig. 5. a Liver prior to subtotal hepatectomy demonstrating numerous small white spots spread over the liver surface 1 day after intrasplenic implantation of 48 × 10^6 hepatocytes (series 4). **b** HE staining of the liver 1 day after intrasplenic injection of 48 × 10^6 primary rat hepatocytes (series 4) demonstrating occlusion of smaller portal branches with consecutive peripheral thromboembolic liver infarction (HE staining, ×200).

Histology

HE staining of the spleen revealed cells characterized as hepatocytes (fig. 4), while histological workup of the lung or peritoneum showed no or no vital hepatocytes, respectively. It is noteworthy that histological examination of the resected liver after intrasplenic implantation of 48 × 10^6 hepatocytes (series 4) revealed macroscopically numerous small white spots at the liver surface (fig. 5a), demonstrating extended occlusion of smaller portal vein branches with consecutive ischemic damage (fig. 5b).

Discussion

When talking about hepatocyte transplantation so far, cell implantation into the portal vein represents the usual technique mimicking clinical trials [14]. Theoretical considerations consist of arguments like the microenvironment after intraportal infusion should be similar to the natural conditions. Consequently, reorganization and interaction with nonparenchymal liver cells are thought to be improved [15].

However, an ectopic site of hepatocyte transplantation may also have advantages over the intraportal implantation route. First, a larger number of hepatocytes may be transplanted to most of the ectopic sites with less invasive techniques and second, less complications may occur than by transplantation through the intraportal route [16]. Moreover, after intraportal implantation portal hypertension might arise, in particular in a model after liver resection or in models with alternated liver architecture, which itself could disturb the potentially positive effects of transplanted cells [17].

Thus, the approach to transplant hepatocytes in an ectopic site may still represent an alternative option if methods can be developed to prolong the survival and functional abilities of these transplanted hepatocytes. In the literature, hepatocyte survival was proven in the following loci: interscapular fat pads, intraperitoneal and subcutaneous tissue (both when attached to an extracellular matrix such as collagen-coated microcarrier beads), and pancreas [10, 18, 19]. When hepatocytes were transplanted into the pulmonary vascular bed or directly into the lung parenchyma, only limited survival of these cells occurred [20]. Additionally, no meaningful hepatocyte survival was observed in skeletal muscle or after intra-arterial infusion into splanchnic or renal vascular beds without using supportive measures like scaffolds [21]. Ohashi et al. [22] showed that hepatocytes can be engrafted in the long term under the kidney capsule. The survival of the transplanted cells was limited, although the results could be improved by cotransplantation of pancreatic islets, which presumably resulted in the local release of hepatotrophic factors [23]. However, studies of the fate of hepatocytes transplanted beneath the renal capsule have been less extensive.

The course of intrasplenically transplanted hepatocytes has been studied most frequently. Kusano and Mito [24] reported that several months after syngenic hepatocyte transplantation, as much as 40% of the spleen was replaced by hepatocytes. In long-term intrasplenic hepatocyte grafts, a hepatic configuration was apparent with bile canaliculi and sinusoids lined with endothelial and fat-storing cells [24]. Furthermore, hepatocytes transplanted into the spleen of rats and mice survived throughout their average life span [21]. Nevertheless, there is a risk if implanting cells into the splenic pulp. It is known that the majority of the implanted cells will translocate to the liver and may occlude portal branches [25]. Even if this is temporary and might not affect a healthy liver environment, in case of preexisting liver damage this procedure could worsen liver function and increase portal hypertension and may negatively influence cell engraftment [26].

As a consequence, we focused our analysis on other sites of implantation in order to avoid irritation of the liver vascular bed. The peritoneum has the advantage of being reached quickly and easily, and serious technical complications following cell implantation are not expected. Since it had already been shown that intraperitoneal implantation has beneficial effects only in combination with special scaffolds [10], we had chosen a technique of subperitoneal implantation of isolated hepatocytes. Hereby, the premise that a technique for cell transplantation should be simple and allow the transplantation of an adequate number of hepatocytes was fulfilled. However, we were not able to show any benefit of this technique in terms of cell or animal survival. Indeed, histological findings revealed only necrotic transplanted hepatocytes along with massive cell decay. An increase in the amount of transplanted cells was not meaningful in view of the histological findings. Thus, the technique of subperitoneal hepatocyte transplantation failed, mostly due to mechanical reasons in favor of cell cluster compression and inability of cell expansion in the peritoneal wall, as we assumed. This is independent of the timing of cell implantation since subperitoneal hepatocyte implantation 1 day prior to surgery also failed to improve the results with death of all animals, except 1, until the third postoperative day.

When changing the implantation technique to an intrasplenic approach, we were surprised that this technique performed on the day of surgery also failed. Indeed, the neurological symptoms which we observed in the treated group of series 3 conformed with portal venous stasis, hypoglycemia, and cerebral edema. Therefore, it was assumed that many of the transplanted cells translocated into the remnant liver, plugged up portal veins, increased the resistance of portal veins, and nearly stopped portal blood flow, thereby aggravating signs of the deleterious small-for-size syndrome [27]. As a consequence, nearly all animals died within the first hours after surgery.

Surprisingly, transplantation of hepatocytes via the intrasplenic approach 1 day before surgery was very effective, resulting in significantly increased animal survival (72 vs. 29% in controls). We believe that following intrasplenic hepatocyte transplantation most of the translocated cells spread on a greater cross section of the portal vascular bed, and obviously, by subtotal hepatectomy 1 day later, most of these translocated cells were removed, too. However, the number of the remaining cells in the spleen and in the remnant liver seemed to still be sufficient to achieve a significant survival benefit.

In the same line of evidence, we interpreted our results of series 4. Undoubtedly, the success of hepatocyte transplantation in view of survival benefit seems to be a matter of cell amount, as demonstrated by 29% survival using no cells, 44% using 16×10^6 cells, or 72% using 24×10^6 cells. Theoretical considerations may confirm an essential limit of cells to overcome hepatic failure in this model. For example, a rat weighing 300 g has about 12 g liver mass (4% of the body weight) [28], and 10% of the functional liver mass was left after the procedure of subtotal hepatectomy. It is supposed that more than 15% of the original liver mass is necessary for transition from hepatic failure to a temporary liver insufficiency [28]. Thus, 5% of the liver mass correspond to 24×10^6 cells, assuming that 1 g liver mass consists of 40×10^6 hepatocytes [29], which was the basic dosage for cell implantation in our study. As a consequence, implantation of this cell amount (24×10^6) should improve postoperative survival as demonstrated in our study. However, a further increase in the amount of transplanted cells (32×10^6 and 48×10^6 cells) did not automatically further improve the postoperative outcome (5-day survival: 38 and 50%, respectively). As shown in figure 5a and b, the increased cell load resulted histologically in the occlusion of smaller portal vein branches. This might have excessively increased the already deleterious signs of the small-for-size syndrome, which may have neutralized the positive effects of hepatocyte cell support.

Although we have established a model of hepatocyte transplantation, there are still questions, especially concerning possibilities of new cell sources, that need to be studied further [30]; moreover, one has to consider that

a cell loading limit has to be respected, due to disturbances of remnant hepatic microcirculation, if the intrasplenic hepatocyte transplantation technique is performed.

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