0041-1337/02/7306-890/0
TRANSPLANTATION
Vol. 73, 890–896, No. 6, March 27, 2002
Printed in U.S.A.
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INTRAPORTAL HEPATOCYTE TRANSPLANTATION IN THE PIG: A HEMODYNAMIC AND HISTOPATHOLOGICAL STUDY[1]

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Background. **Hepatocyte transplantation is an attractive treatment for various liver diseases. The intraportal route of transplantation is favored, but little information is available on the possible adverse effects in this technique. We investigated the influence of intraportal loads of hepatocytes on portal, pulmonary, and systemic hemodynamics in 13 pigs.**

Methods. **Under general anesthesia, pigs were provided with an arterial line, a Swan-Ganz catheter, and two intraportal catheters, one for cell infusion and one for heparin infusion and portal pressure measurement. Pig hepatocytes were infused at a rate of 25 million cells/min.**

Results. **The first six animals were used to develop the infusion technique. In the last seven animals, portal pressure increased linearly with cell load upon infusion of 400–2400×10^6 hepatocytes (r^2=0.704; P<0.05). Portal flow measured by Doppler sonography decreased by 23–66% below basal values. An inverse linear relationship was found between portal pressure and portal flow (r^2=0.679; P<0.05), portal flow approaching zero for portal pressure >40 mmHg. Pulmonary arterial pressure increased by 11–62%. AST increased up to 10-fold, and platelets decreased by 22–58%. Hepatocytes-containing thrombi were present in segmental and in smaller portal branches. Hepatocytes were always identified in lung sinusoids 48 hr after infusion, and a small basal pulmonary infarction was found in one animal.**

Conclusions. **These data suggest that up to 2.4% of total hepatocyte mass can be infused in this large animal model. However, the risk of significant thrombotic complications should be considered for clinical applications.**

Hepatocyte transplantation has a potential to become an alternative to whole liver transplantation for the treatment of inborn errors of liver metabolism and of acute liver failure (*1–6*). The critical issues that must be addressed before this promising therapeutic modality is to be recommended for wide clinical use are (a) the number of cells needed to achieve therapeutic goals, and (b) how many cells can be transplanted safely, either once or repeatedly. Most investigators favor the intraportal route of hepatocyte transplantation, because the cells are placed in a physiological environment (*7–15*). Furthermore, it has been shown by many authors that hepatocytes infused into the portal vein integrate with hepatic cell plates without altering liver micro-architecture (*16–19*). However, despite growing experience with intraportal hepatocyte transplantation in humans, the risks related to this particular procedure have not been fully appreciated. In particular, the hemodynamic and anatomical sequelae of intraportal hepatocyte infusion have not been studied systematically, even though reports on massive portal thrombosis and/or pulmonary embolism in experimental animals and humans date back to the 1970s (*7*, *11*, *20*, *21*).

In the present work, we investigated the portal, pulmonary, and systemic hemodynamic effects of intraportal hepatocyte transplantation in normal pigs. The effects of cell transplantation on liver and lung morphology were also studied. The transplantation technique was developed in this large animal model to reproduce as closely as possible the condition of clinical cell therapy.

MATERIALS AND METHODS

Animals

Twenty-six Landrace pigs (body weight 29–36 kg; liver weight 810–980 g) were used in this study. Thirteen animals were used as donors for hepatocyte isolation and 13 animals as recipients. All procedures on the animals were performed under general anesthesia. This was induced with intramuscular (i.m.) ketamine (2 mg/kg body weight) and xylazine (6 mg/kg body weight) plus i.v. thiopental (5 mg/kg body weight) and maintained with isoflurane in air/oxygen mixture plus Fentanyl as needed. The animals received human care according to NIH criteria (NIH publication 86-23 revised 1985).

Experimental Design

A first series of six pigs was used to develop a suitable technique for intraportal infusion of hepatocytes in this animal model, focusing in particular on the effects of heparin and on techniques for the placement of catheters. In a second series of seven animals, increasing amounts of hepatocytes were transplanted with evaluation both of hemodynamic and of biochemical parameters. Forty-eight hours after the infusion of hepatocytes, the pigs were killed and subjected to autopsy. The liver and the lungs were excised and inspected through multiple sections 1-cm wide. Both areas of suspected lesions and areas in identical regions were sampled for histological examination.

[1] Supported by grants from the Regione del Veneto and the Fondazione per l'Incremento dei Trapianti d'Organo, Padova, Italy.

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Isolation of Hepatocytes

Cell isolation was performed with a modification of the Seglen collagenase digestion technique (*22*). The whole liver was first perfused with Ca^{2+}/Mg^{2+}-free HEPES buffer adjusted to pH 7.4 until the perfusate was clear of erythrocytes. The liver was then perfused with 500 ml of HEPES (Boehringer Mannheim, Germany) buffer, pH 7.6, containing collagenase B (0.1% w/v) and 10 mM of $CaCl_2$ and subsequently with 1000 ml of HEPES buffer, pH 7.6, containing collagenase B (0.05% w/v) and 20 mM of $CaCl_2$ for 10–15 min in a recirculation system. All solutions were warmed at 37°C and gassed with 100% O_2. The digested tissue was gently disrupted with fingers, filtered through a series of 450 μm, 250 μm, and 150 μm filters to remove cell aggregates, suspended in ice-cold William's E medium and centrifuged at 50 g for 5 min. After resuspension in Ringer lactate solution (pH adjusted to 7.4), cells were counted and viability was in the range of 87–96%, as determined by trypan blue exclusion. The final single cell suspensions of 1.5×10^6 cells/ml in 500- or 800-ml plastic bags were kept on ice until transplantation for a maximum of 6 hr. No significant change in viability was noticed during this period of time.

Hepatocyte Transplantation

An arterial line was placed in the right carotid artery, and a flow-directed catheter was introduced into the pulmonary artery through the external jugular vein. The intravascular catheters were connected to pressure transducers linked to a four-channel polygraph (Siemens SC 9000 Hemo-4, Medicinsk Teknik, Solna, Sweden) for continuous monitoring of systemic and pulmonary pressures. Cardiac output and associated parameters were measured by a thermodilution technique (Oxymetrix 3, Abbott Laboratories, Chicago, IL). The abdomen was then accessed through a midline laparotomy. A tributary of the superior mesenteric vein was cannulated with a PVC CH 06 catheter (e.d. 2.0 mm, Maersk Medical, Hundestedt, Denmark), which was fed into the portal vein. A second CH 05 catheter (e.d. 1.7 mm) was introduced into a second small tributary of the superior mesenteric vein and pushed just proximal to the first one. The first catheter was used for hepatocyte infusion, while the second catheter was used for independent heparin infusion at a rate of 20 UI/kg/hr and for portal pressure monitoring by connecting it to the pressure transducer via a three-way stopcock. Portal flow was measured by percutaneous Doppler sonography. After a control infusion of 250–500 ml of Ringer lactate solution without cells, hepatocyte infusion was performed by gravity at a rate of approximately 25 million cells/min in batches of $400-800\times10^6$ cells using a red blood cells transfusion filter. All hemodynamic parameters were recorded at the following time points: 0 (pre-infusion), 15 min after the beginning of infusion, end of infusion, and 15, 30, and 45 min after the end of infusion. Blood samples were taken in basal conditions and at the end of the infusion for determination of blood cell count, prothrombin time, partial thromboplastin time, fibrinogen degradation product D-dimer, and aspartate aminotransferase (AST).

Morphological Studies

For histological analysis, liver and lung samples were fixed in 10% formalin and embedded in paraffin. The tissues were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), PAS-diastase, and Van Gieson. Hepatocytes were identified in the lung by immunohistochemistry, using a monoclonal mouse anti-human hepatocyte antibody (OCH1E5, Dako, Glostrup, Denmark). Positive controls were performed on pig and human liver sections, and a specific cross-reactivity was observed only for hepatocytes. Negative controls were performed by replacing the primary antibody with a nonimmune serum on pig and human liver sections and on pig lung sections. No reactivity was found.

Statistical Analysis

Values are expressed as mean±SD. A SigmaStat microcomputer program (Jandel Scientifics, San Rafael, CA) was used to evaluate differences between groups with the Wilcoxon signed rank test and for regression analyses. A P value <0.05 was considered statistically significant.

RESULTS

Development of a Technique for Intraportal Infusion of Hepatocytes

In a first series of experiments, heparin was added to the cell suspension at a final concentration of 10 U/ml. This resulted in the formation of cell microaggregates, which upon infusion of as little as 400 million cells produced massive portal thrombosis. Omission of heparin resulted in a pure single-cell suspension, but infusion in the absence of anticoagulant also resulted in rapid vascular thrombosis. Finally, the use of two separate catheters for cell and for heparin infusion allowed transplantation of large amounts of cells without obvious development of vascular complications.

Hemodynamics

No changes in either portal or pulmonary pressure were observed during control infusions of Ringer's lactate without cells. In seven animals, hepatocyte transplantation produced an increase of portal pressure that was directly related to the amount of cells infused ($r^2=0.704$; $P<0.05$) up to four times basal values (Fig. 1). Three of these animals received a single infusion of cells (400, 600, or 800×10^6 hepatocytes), whereas the last four pigs received multiple infusions (up to 2.4 billion hepatocytes in three batches of 800×10^6 cells each). The behavior of portal pressure in these four animals is shown in Figure 2. Intraportal cell transplantation resulted in a rise of portal pressure, which decreased slowly after cessation of infusion and then reached a plateau after 45 min. In the first set of experiments, portal pressure was monitored up to 2 hr

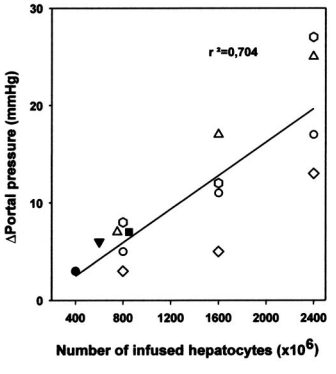

FIGURE 1. Relationship between portal pressure and number of infused hepatocytes in seven pigs. Hepatocyte transplantation induced an increase of portal pressure that was directly related to the amount of cells infused up to four times basal values. Each symbol refers to values recorded in a single animal. Three animals (filled symbols) received a single infusion of 400, 600, or 800×10^6 cells, respectively, while four animals (open symbols) received three subsequent infusions of 800×10^6 cells each, up to a total of 2.4×10^7 cells. Portal pressure was recorded at the end of each batch infusion.

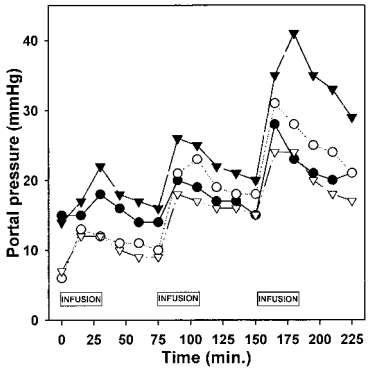

FIGURE 2. Changes of portal pressure during hepatocyte transplantation. Intraportal cell transplantation in four pigs receiving three batches of hepatocyte suspension (800×10^6 cells per batch) resulted in a rise of portal pressure, which decreased slowly after cessation of infusion and reached a plateau after 45 min.

after each infusion. We observed that pressure values tended to level off after this time period, without returning to baseline. Longer observation times were not compatible with the experimental set-up in animals under general anesthesia. Therefore, in the second set of experiments, the intervals between infusions were kept at 45 min. Portal flow was measured in the four animals receiving multiple infusions and it decreased by 23–66% below basal values. An inverse linear relationship was found between portal pressure and portal flow ($r^2=0.679$; $P<0.05$) (Fig. 3), the latter approaching zero when portal pressure exceeded 40 mmHg. Pulmonary arterial pressure increased after cell infusion by 11–62% (mean=29%) of basal values. However, no relationship was found between the amount of infused cells and the increase of pulmonary arterial pressure (Fig. 4). Pulmonary capillary wedge pressure, mean arterial pressure, central venous pressure, and cardiac output did not change significantly throughout the procedure (Table 1).

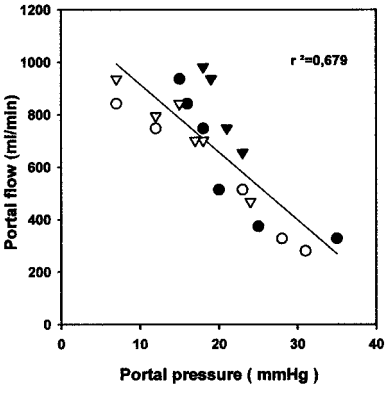

FIGURE 3. Relationship between portal pressure and portal flow in the four pigs infused with increasing loads of hepatocytes. Each animal received three subsequent infusions of 800×10^6 cells each, up to a total of 2.4×10^7 cells. Both portal pressure and portal flow were recorder after each batch infusion. An inverse relationship was found between portal pressure and portal flow, the latter approaching zero when portal pressure exceeded 40 mmHg.

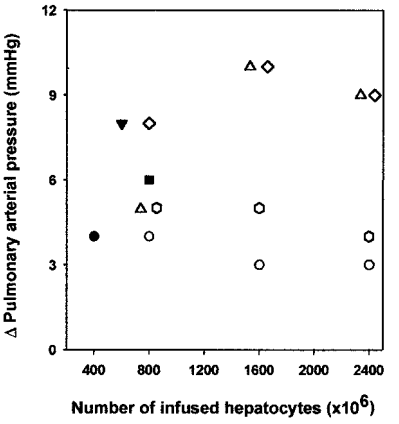

FIGURE 4. Effect of infusion of different amounts of hepatocytes on pulmonary arterial pressure. Each symbol refers to values recorded in a single animal (n=7). Three animals (filled symbols) received a single infusion of 400, 600, or 800×10^6 cells respectively, while four animals (open symbols) received three subsequent infusions of 800×10^6 cells each, up to a total of 2.4×10^7 cells. No relationship was found between the amount of infused cells and the increase of pulmonary arterial pressure.

Biochemical Parameters

AST increased about four times after hepatocyte infusion. Platelet count decreased in all animals by 22–58%. No significant change of the remaining biochemical parameters was observed (Table 2).

Liver and Lung Pathology

Small thrombi containing hepatocytes were found in many segmental and subsegmental branches of the portal vein (Fig. 5). Nonetheless, on histological examination the liver parenchyma showed no abnormal changes (Fig. 5). Clusters of hepatocytes were present in the pulmonary septal capillaries 48 hr after transplantation (Fig. 6) as confirmed by immunohistochemistry (Fig. 7). A 1.5-cm basal pulmonary infarction was found and documented by light microscopy in one pig (Fig. 8).

DISCUSSION

The possibility to treat effectively hepatic inborn errors of metabolism with hepatocyte transplantation rather than with whole liver transplantation has been demonstrated in experimental animals (*1*, *4*, *6*) and in a limited number of patients (*23*, *24*). Even if intrasplenic hepatocyte transplantation has been performed in patients with acute liver failure (*24–28*), the intraportal route has always been preferred for the treatment of metabolic disorders (*23–25*), because it is believed that integration of transplanted hepatocytes with hepatic cell plates is a prerequisite for optimal long-term maintenance of function (*1*, *6*). Previous reports focused primarily on clinical outcome and biochemical parameters but did not describe accurately changes in portal and pulmonary hemodynamics associated with cell infusion. In addition, data in laboratory animals are scarce and conflicting. Portal thrombosis was reported after intraportal hepatocyte transplantation in beagles (*20*). In two dalmatian dogs, receiving 1 billion hepatocytes, there was a fourfold increase in portal

TABLE 1. Hemodynamic parameters before and after intraportal hepatocyte infusion (mean±SD; n=7)

Parameter	Basal	After cell infusion
Mean arterial pressure (mmHg)	82.8±18.3	78.7±10.2
Central venous pressure (mmHg)	10.1±7.3	9.9±5.5
Pulmonary capillary wedge pressure (mmHg)	11.8±2.9	12.2±4.0
Cardiac output (L/min)	5.3±2.0	6.4±1.8

TABLE 2. Effect of intraportal hepatocyte infusion on some biochemical parameters (mean±SD; n=7)

Parameter	Basal	Middle of cell infusion	After last cell infusion
Leucocytes ($\times 10^6$/L)	10530±1813	11077±2142	10691±2599
Hemoglobin (g/L)	88.7±7.8	89.1±7.6	83.9±14.1
Hematocrit (%)	26.7±2.1	27.0±2.1	24.7±4.0
Platelets ($\times 10^9$/L)	395.3±89.6	292.6±83.1	223.5±59.8[a]
PT (%)	96.1±8.4	89.6±6.8	88.2±10.5
APTT (sec)	17.6±7.7	19.4±8.1	18.9±4.5
FDP D-Dimer (μg/L)	96.6±48.0	112.7±53.7	124.8±45.4
AST (U/L)	40.3±8.1	90.0±19.8[a]	163.5±101.4[a]

[a] Different from basal values, $P<0.01$.

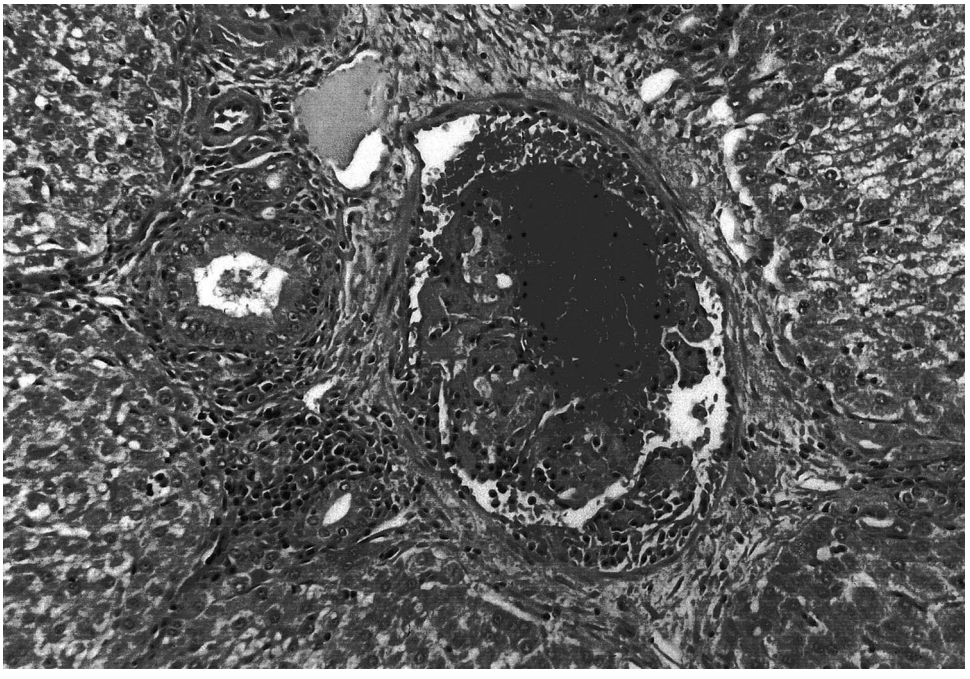

FIGURE 5. Histological analysis of liver parenchyma reveals hepatocyte-containing thrombi occluding a portal vein. Note the normal appearance of the surrounding hepatic parenchyma. (H&E, ×20).

pressure and portal flow decreased by about 70% (*11*). A similar rise in portal pressure was observed in a monkey receiving 0.64×10^9 hepatocytes, but in another monkey infused with 1.09×10^9 hepatocytes the increase in portal pressure was only 34%. In a third animal transplanted with 1.97×10^9 cells, portal pressure actually decreased after cell infusion, perhaps due to technical problems (*29*). The present study demonstrates that in seven pigs the increase in portal pressure was proportional to the number of transplanted hepatocytes. Furthermore, portal pressure values were inversely related to portal flow, suggesting that during cell infusion the rise of pressure above 40 mmHg can stop the portal flow. This was observed in some animals in the first series of experiments. How can these data be reconciled with previous reports and extrapolated to the clinical situation in humans? In published studies, the number of hepatocytes infused into the portal vein was usually expressed as percentage of total hepatocyte mass in the native liver. The values ranged from 0.1% to 2% in rats (*8*, *12*, *15*, *30–31*), from 1% to 2% in rabbits (*14*, *32–34*), from 2.0% to 4.4% in dogs (*11*, *20*), and from 0.75% to 2.4% in monkeys (*29*). However, it has been suggested that the liver can accommodate up to 10% of total hepatocyte mass (*35*). When these data are expressed as the number of transplanted cells per 1 g of native liver weight, then the above variability is reduced, and one could argue that the maximal number of hepatocytes per 1 g of liver that could be safely infused are $2.1–2.9\times10^6$ in rats (*8*, *15*), 2×10^6 in rabbits (*14*, *34*), 2.7×10^6 in Dalmatian dogs (*11*), and 3.3×10^6 in monkeys (*29*). Based on the present study, the corresponding value for pigs would be 2.4×10^6 cells/g liver. In six reported cases of human hepatocyte transplantation, the number of cells infused into the portal vein

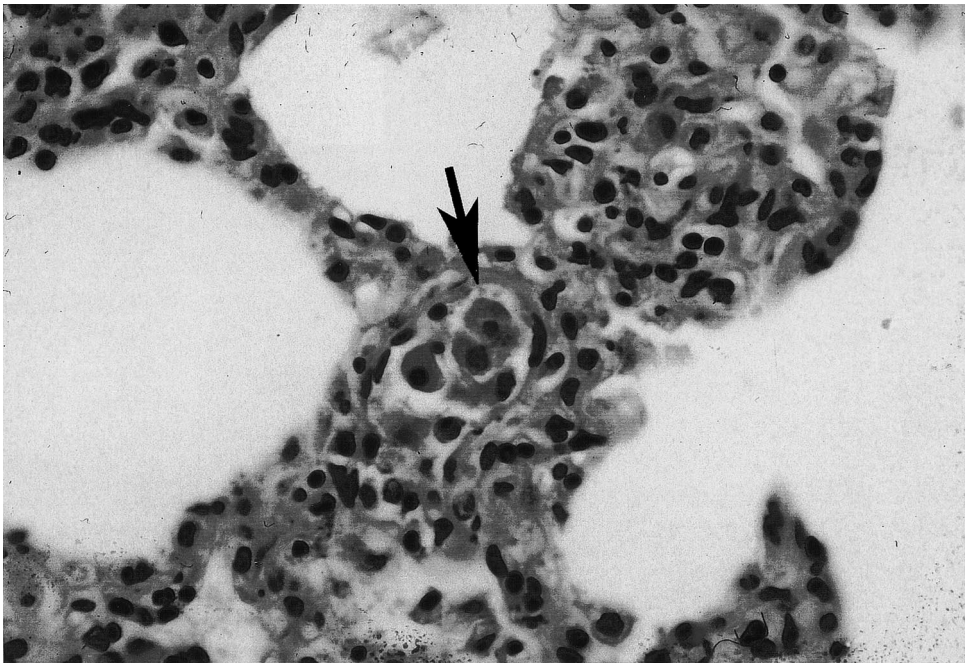

FIGURE 6. Histological analysis of a lung sample. The section of tissue shows clusters of hepatocytes present in lung capillaries (arrow) 48 hr after intraportal infusion (H&E, ×40).

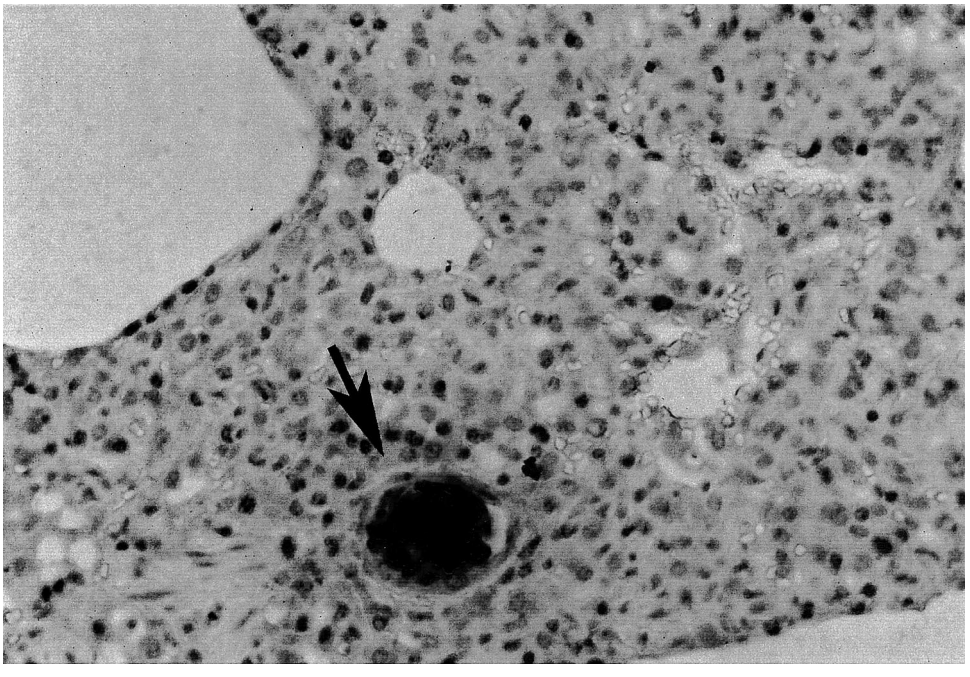

FIGURE 7. Immunohistochemical staining with a monoclonal mouse anti-human hepatocyte antibody of a lung samples. The section of tissue shows positive-staining hepatocytes in lung capillaries (arrow) 48 hr after intraportal infusion. (H&E, ×40).

ranged from 10×10^3 to 400×10^3 (*3*, *25*, *28*). Soriano et al. (*28*) and Fox et al. (*24*) infused up to 14×10^6 hepatocytes per 1 g of liver over 4 days in a 3-year-old boy and over 15 hr in a 10-year-old girl, respectively. It is not known whether the young age of the recipients allowed transplantation of large hepatocyte mass without clinically apparent complications. Bilir et al. (*21*) reported both intraportal and intrasplenic infusion of hepatocytes in two adult patients. A total of 10×10^9 and 30×10^9 cells were transplanted, but the relative number of cells infused into the portal vein was not specified. In summary, the results of this study together with published data on hepatocellular transplantation in different animal species suggest that up to about $2.5–3\times10^6$ hepatocytes per g of liver can be infused into the portal vein with reasonable safety. Assuming that 1 g of liver contains 100×10^6 hepatocytes (*20*), this number of cells corresponds to approximately 2 to 3% of total hepatocyte mass, and therefore, in most clinical cases it should have a therapeutic effect (*3*, *6*). Although transplantation of a greater number of cells should be theoretically more effective, seeding of large hepatocyte clusters in hepatic sinusoids could cause or aggravate hypoxic damage to transplanted cells. In fact, more viable hepatocytes were seen in clusters containing 1–6 cells than in clusters comprised of more than 6 cells (*18*). Several hepatocyte-containing thrombi were observed in the small branches of the portal vein. This finding could explain the

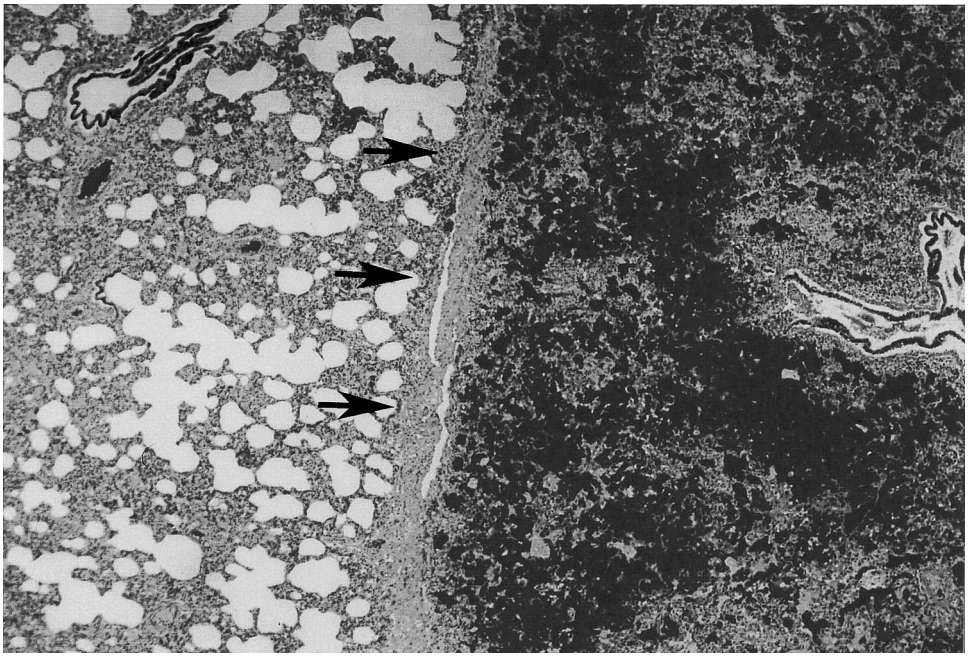

FIGURE 8. Histological analysis of a lung sample. A lung infarction (arrows) is documented by light microscopy in the inferior left lobe of one pig infused with a total of 2.4×10^9 hepatocytes via the portal vein (H&E, ×4).

decrease in platelet count after cell infusion, but it did not result in damage to the surrounding hepatic parenchyma, probably due to a compensatory contribution of arterial blood flow.

To our knowledge, the ability of heparin to favor the formation of hepatocyte aggregates in vitro was not reported previously. However, we did not observe the same effect with rat and human hepatocytes. The nature of this apparently species-specific effect remains unknown.

It is well established that some of the transplanted hepatocytes migrate from the liver into the lungs, where they are entrapped due to the smaller size of pulmonary capillaries (*36*, *37*). Surprisingly, no reports are available on the effects of hepatocyte transplantation on pulmonary circulation. The present data indicate that migration of hepatocytes to the lung can result in lung infarction. The increase in pulmonary arterial pressure after hepatocyte transplantation suggests an increase in pulmonary resistance secondary to obstruction of small pulmonary vessels. Because the increase in pulmonary artery pressure did not correlate with the amount of cells infused, one could argue that after initial translocation, additional cells infused became entrapped in liver sinusoids and portal venules rather than escaping to the lungs. It is also possible that local vasoconstriction contributed to the observed increase in pulmonary arterial pressure after hepatocyte transplantation. Whatever the underlying mechanism, the risk of pulmonary hypertension and embolization must be considered whenever hepatocyte transplantation is attempted in the clinical setting. Pulmonary embolism has been reported in rats (*36*, *37*) and more recently in patients with acute liver failure (*21*). It is worth noting that in the latter report, the presence of portosystemic shunting (*38*, *39*) could have been a factor behind massive embolization. In rats, radiolabeled hepatocytes were cleared from the lung capillaries within 24 hr after transplantation (*37*). Similarly, Bilir et al. (*21*) found that intraportal hepatocyte transplantation in patients with acute liver failure was followed by hypoxemia and pulmonary infiltrates in chest radiograph, which both improved after 24 hr, suggesting that most of the cells had been cleared from the lungs by that time. It is noteworthy that in these patients no lung infarction was found at autopsy despite massive hepatocyte embolization in pulmonary capillaries. A possible explanation is that the coagulation defect typical of severe liver failure could have prevented the development of thrombosis. In the present study, transplanted hepatocytes were found in all lungs studied after 48 hr. At the dosage used, heparin infusion did not prolong significantly partial thromboplastin time, and one animal developed a small basal lung infarction. These data suggest that, in the absence of severe liver disease, an appropriate anticoagulation therapy should be considered for clinical hepatocyte transplantation.

In conclusion, this work represents an effort to provide guidelines for intraportal hepatocyte transplantation by calculating the maximal number of cells that can be safely infused into the liver. The incidence of significant complications after the procedure in this animal model suggests that patients undergoing intraportal hepatocyte transplantation should be carefully monitored for the possible occurrence of both intraportal and pulmonary thrombosis.

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Received 19 April 2001.
Revision Requested 12 July 2001.
Accepted 28 September 2001.