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HETEROTOPIC AUXILIARY LIVER IN AN ISOLATED AND VASCULARIZED SEGMENT OF THE SMALL INTESTINE IN RATS[1]

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Background. **Development of an auxiliary liver is of interest for treating several conditions. To examine whether an isolated intestinal segment will support development of a heterotopic auxiliary liver, we studied the fate of liver microfragments in rats.**

Methods. **Small intestinal segments with intact circulation were created, and the small intestinal mucosa was removed. The intestinal segments were filled with autologous liver microfragments, and animals were studied for various periods.**

Results. **Initially, liver microfragments were engulfed by a serosanguineous exudate enriched in polymorphonuclear leukocytes, suggesting an early granulation-type response. Transplanted liver fragments were subsequently reorganized and showed morphologic integrity with typical hepatic lobular organization. Transplanted tissue contained healthy hepatocytes with abundant glycogen content. Transplanted liver remained intact in the small intestine for up to 40 days, although at later times portal fibrosis and bile ductular proliferation were apparent, despite the absence of cholestasis or hepatocellular abnormalities. In contrast, instillation of liver microfragments in the peritoneal cavity led to rapid loss of tissue integrity and phagocytotic clearance of transplanted tissue.**

Conclusions. **Small intestinal segments denuded of the mucosal layer can support heterotopically transplanted liver. Further development of this auxiliary liver system will provide insights into mechanisms concerning neo-organogenesis and into potential therapeutic applications of heterotopic liver in specific diseases.**

Orthotopic liver transplantation (OLT) is limited in many parts of the world by shortages or nonavailability of donor livers. Alternatives to OLT include bioartificial liver-assist devices and cell therapy, although far more work is needed for meeting the promise of these modalities (*1–3*). In conditions in which the liver can potentially recover, for example, acute liver failure, OLT can be lifesaving but constitutes an "irreversible procedure." Application of OLT to correct metabolic deficiency states manifesting without liver disease, such as Crigler-Najjar syndrome and familial hypercholesterolemia (*4*), is less desirable in view of its irreversibility. In these situations, and also in acute liver failure, auxiliary liver transplantation represents an attractive alternative to OLT (*5–11*). For instance, an auxiliary liver could provide hepatic function without the loss of the native liver. However, various studies established that auxiliary livers may atrophy in the absence of portal blood supply, which contains putative hepatotropic factors (*12,13*). This led to the development of auxiliary partial OLT, in which the portal blood supply is allocated to both transplanted and native livers, with more efficient graft survival (*5–11*). Despite these advances in liver transplantation, creation of an auxiliary liver in heterotopic sites remains an attractive concept. Continuing advances in stem cell biology, extracellular matrix biology, and tissue remodeling offer the potential for testing specific concepts relevant for such neo-organogenesis.

We reasoned that investigation of suitable anatomic sites that help support revascularization of transplanted liver tissue and thus favor the establishment of an heterotopic organ would be appropriate. It is now well established that transplanted hepatocytes can survive for variable periods in multiple heterologous sites, such as the spleen, peritoneal cavity, and mesenteric laminates of the small intestine (*14–16*). On the other hand, isolation of liver cells leads to the loss of acinar organization, which could be deleterious, whereas tissue organization is not totally disrupted in liver fragments or liver slices. However, in early studies, transplantation of large liver fragments in extrahepatic sites, including the anterior chamber of the eye, subcutaneously, or next to the liver capsule, was ineffective (reviewed in 17). We considered that liver microfragments should survive in ectopic locations if superior opportunities were provided for oxygenation, mass transfer, and revascularization. Here, we present our early experience with the small intestine as a suitable location for establishing heterotopic liver. Review of the intestinal anatomy suggested to us that a vascularized segment could be isolated without compromising its viability or integrity. We ablated the small intestinal mucosa of this isolated segment to bring liver microfragments closer to the submucosal intestinal vessels. The larger surface area of liver microfragments compared with whole liver of an equivalent size should have facilitated gas and mass exchange during the process of revascularization and tissue remodeling. Our studies show that these principles were successful in producing heterotopic auxiliary liver in intestine segments.

MATERIALS AND METHODS

Animals

After pilot studies, final experiments used 60 outbred Wistar rats weighing 150 to 250 g. Animals were housed in standard laboratory

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conditions under 12-hr day-night cycles with provision of pelleted rodent diet and water ad libitum. All animals received care according to institutional guidelines.

Animals were provided only water for 1 day before surgery. All surgical procedures were under ether anesthesia using aseptic techniques. One group of rats (n=30) received liver microfragments in small intestine segments (see later). In a second group of rats (n=30), liver fragments were injected into the peritoneal cavity by a mini-laparotomy.

Preparation of Liver Microfragments

Because our animals were outbred, donor organs were harvested from individual animals to avoid the problems of allorejection. The left lateral liver lobe, constituting 35% of the liver mass, was removed according to the standard Higgins and Anderson partial hepatectomy method. The liver was flushed through the portal vein branch with sterile oxygenated Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO) at 28°C, minced with a surgical knife into fragments of 1 mm^3 or smaller size in Eagle's minimal essential medium (Sigma), and passed through two layers of surgical gauze to eliminate large tissue fragments. Subsequently, liver microfragments were stored at 28°C in minimal essential medium until transplantation.

Creation of Intestinal Heterotopic Liver

The ileum was inspected to select 1- to 2-cm long segments with intact arteriovenous supply. Ligatures of 3-0 nylon or silk were placed proximally and distally, and the isolated intestinal segment was then inverted inside out by grasping with anatomical forceps. A number 10 surgical scalpel blade was used to scarify the intestinal mucosa of the inverted segment until complete denudation. The segment was irrigated with normal saline and restored to its normal inside-in position. One end of the separated intestinal segment was then closed with 3-0 nylon or silk thread. The intestinal segment was filled to capacity with liver microfragments using a syringe. Intestinal integrity was restored by side-to-side entero-entero anastomosis using 7-0 surgical silk. The segment containing liver fragments was anchored to adjacent mesentery with a serosal suture using 6-0 or 7-0 nylon or Vicryl. After abdominal closure, animals were kept warm until recovery from anesthesia.

Rats were killed in groups of two to three animals at 2 hr and at 1, 4, 10, 12, 16, 28, 35, and 40 days after surgery. The experiments were repeated up to four times to verify key findings.

Histologic Studies

Tissues were fixed in formalin and embedded in paraffin according to standard procedures. Tissue analysis focused on morphology of transplanted liver and integrity of the auxiliary liver in the intestine. The host response was analyzed by studying the nature of cellular infiltrates. Apoptosis was analyzed by examining chromatin condensation, nuclear fragmentation, and areas of cell loss. Assessment of hepatocyte morphology included analysis of hydropic changes or steatosis, which was further verified by Sudan 3 or Sudan black stains using standard methods.

To demonstrate liver-specific function, tissues were stained for glycogen. Five- to 6-μm-thick sections were fixed in ethanol-acetic acid mixture (99:1 v/v), washed in distilled water, and incubated with 1% aqueous periodic acid for 5 min. After rinsing in water, sections were incubated in Schiff reagent for 5 min and washed three times in 0.5% sodium bisulfate in water followed by washes in running tap water for 10 min. The intensity of glycogen staining was graded from 0 (absent), 1+ (low expression), 2+ (moderate expression), and 3+ (maximal expression observed in the normal liver).

Statistical Analysis

Where appropriate, data are expressed as means ± SD. Statistical comparisons were not needed for the data presented.

RESULTS

Animal Outcomes Following Surgery

The entire surgical procedure to create the intestinal liver required approximately 40 min. Rats recovered promptly after surgery. All animals survived after implantation of liver microfragments in the peritoneal cavity. On the other hand, animals subjected to surgery for auxiliary liver showed occasional mortalities after wound infection, internal bleeding, or intra-abdominal abscesses. With experience, this mortality decreased to approximately 10% of the operated animals.

Development of Auxiliary Liver in the Intestine

The critical components of the surgical procedures are shown in Figure 1. The typical length of the intestinal segment used for the auxiliary liver was 2 cm with an outside diameter of 4 mm. The weight of liver fragments placed in isolated bowel segments averaged 0.3 g. Comparison with the weight of the donor liver indicated that the initial mass of the auxiliary liver approached 4% to 6%. The overall weight of the auxiliary liver did not decrease subsequently, and in fact, was slightly greater, perhaps because of the presence of additional blood and stromal tissue (P=NS). At necropsy, the small intestine was grossly normal without evidence of gastrointestinal obstruction. The intestinal segment containing transplanted liver tissue appeared healthy, with dark red-brown color. Transplanted liver fragments showed normal

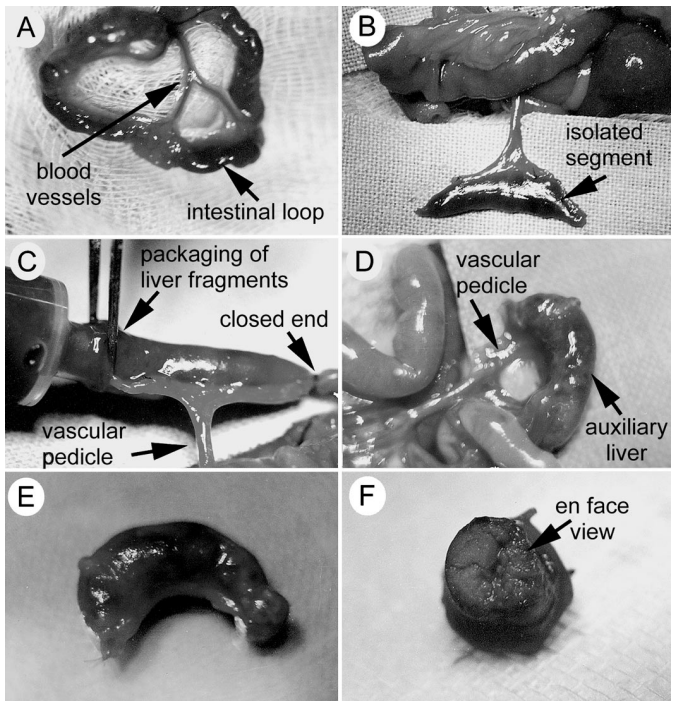

FIGURE 1. Creation of an auxiliary liver in the intestine. (A) Ileal segment with satisfactory vascular pedicle. Isolation of segment in A is shown in B. (C) Instillation of liver microfragments in the isolated intestinal segment. A ligature closed one end of the segment. (D) Completion of auxiliary liver. (E and F) Appearance of healthy auxiliary liver 4 weeks later, with a cross-sectional view of the liver in F.

liver color and consistency. The intestinal wall was adherent loosely to the transplanted liver tissue.

Histologic examination of the auxiliary liver showed complete denudation of the intestinal mucosa down to the submucosa. Liver fragments were enclosed within this denuded intestinal segment, separated initially from the submucosa by an extensive serosanguineous exudate (Fig. 2A). Transplanted liver appeared healthy at early times between 2 hr and 1 day after transplantation, with lobular integrity shown by intact portal tracts, and absence of tissue destruction (Fig. 2B). However, the onset of a granulocytic response was observed in transplanted tissues within 2 hr after surgery. The next stage of tissue reorganization started 2 to 3 days after liver transplantation. Liver fragments became coalesced into continuous sheets of liver tissue. Although transplanted liver fragments remained separated at places from the intestinal submucosa, with hemorrhagic exudates persisting for up to 12 days after liver transplantation (Fig. 2C), the parenchyma of transplanted liver fragments appeared to be normal without cellular disorganization, steatosis, or hydropic changes. These findings were possibly in agreement with revascularization of the engrafted liver tissue, although further analysis was required.

The inflammatory cell infiltrate surrounding transplanted liver fragments maximized 4 days after surgery. More than 95% of the cells in inflammatory infiltrates were neutrophils with round mononuclear cells constituting the remainder, which was in agreement with phagocytic responses. Inflammation subsided subsequently, and infiltrating cells were no longer observed 12 days after surgery. Subsequent to the

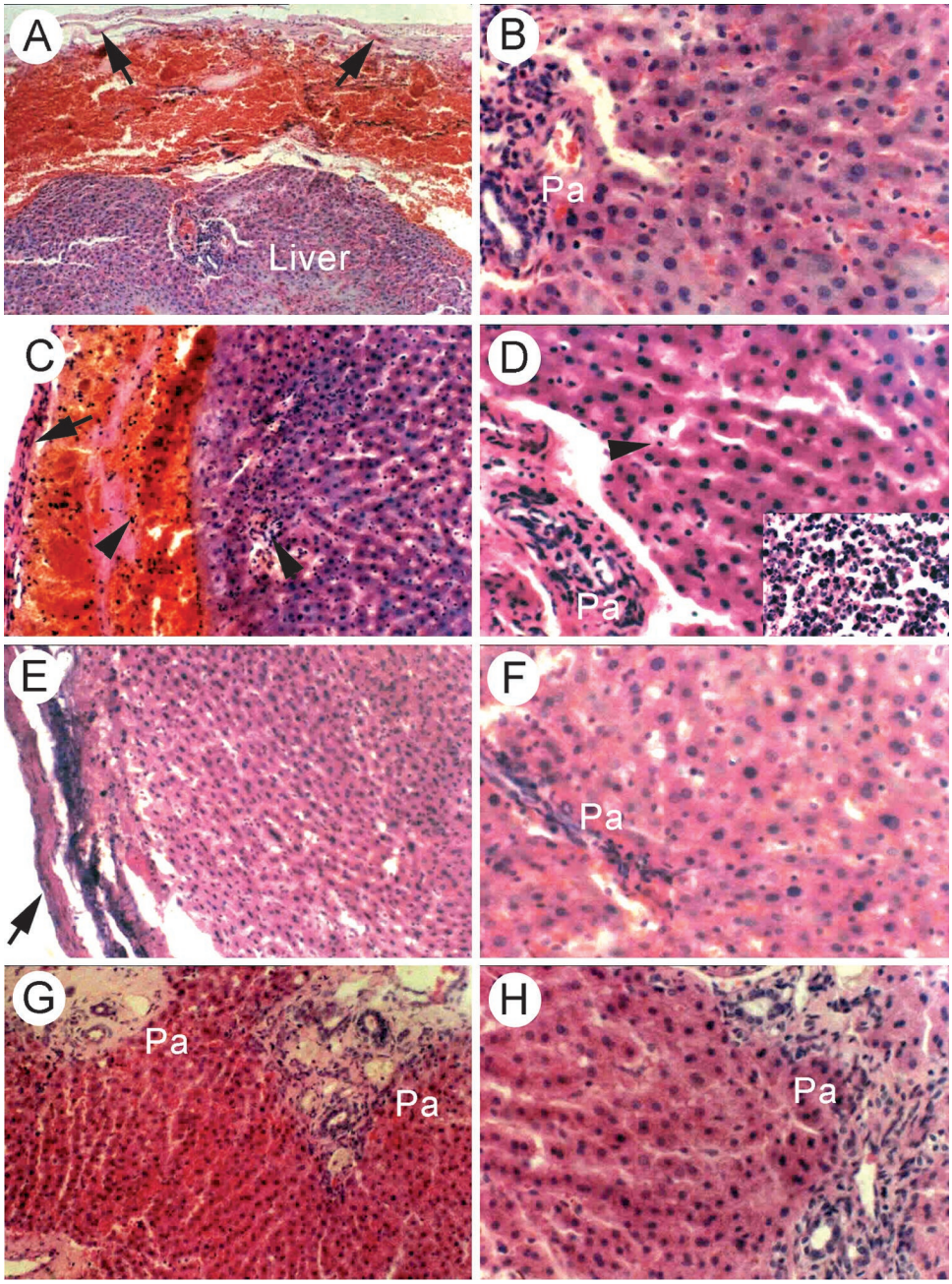

FIGURE 2. Histologic appearance of transplanted liver fragments. (A) Transplanted fragments in the small intestinal segment shortly after completion of surgery with extensive hemorrhagic exudates. Note that the mucosa is completely denuded from the intestinal wall (*arrows*). (B) Liver fragments showing healthy hepatocytes 1 day after transplantation. Pa, portal area. (C) Liver fragments showing reorganization after 7 days, although significant separation is obvious between the intestinal wall (*arrow*) and liver fragments with significant hemorrhagic exudates (*arrowhead*). (D) A higher magnification view of tissue in C with infiltrating phagocytes (*arrowhead*), although neutrophilic infiltration of tissues was far more pronounced after 4 days (*inset*). (E) Liver fragments showing extensive remodeling after 3 weeks, with disappearance of the inflammatory exudate, thickening of the intestinal wall with fibrosis (*arrow*), and normal, healthy-appearing hepatocytes (F). (G and H) Healthy-appearing hepatocytes along with expansion of portal areas (Pa) showing biliary proliferation and portal fibrosis. (Magnification: A, ×100; C, E, and G, ×200; B, D, F, and H, ×400.)

third week after liver transplantation, the intestinal segment underwent further reorganization with disappearance of hemorrhagic exudates and accumulation of fibrous tissues in the intestinal submucosa (Fig. 2E,F). The transplanted liver parenchyma was devoid of focal hepatitis or cholangitis, as might have occurred after infection from normally resident intestinal microbes. We did not observe involution of the liver during remodeling of transplanted fragments, and hepatocyte apoptosis was rare. Also, hepatocytes were not observed to be in mitosis. However, we observed portal fibrosis accompanied with biliary proliferation in liver fragments 28 days after transplantation (Fig. 2G,H). Some areas of transplanted liver fragments were engulfed by fibrosis extending from the underlying intestinal layer. Nonetheless, the transplanted liver parenchyma was mostly intact, and hepatocytes showed normal morphology with maintenance of liver cord structure in the absence of overt sinusoidal fibrosis (Fig. 2H). These findings remained unchanged in some animals during subsequent periods of observation up to 40 days after liver transplantation.

Transplanted liver fragments showed the presence of readily stained glycogen in hepatocytes (range 2+ to 3+). Glycogen content decreased in perivenous hepatocytes at early times (2–4 days) after liver transplantation, but the normal pattern of glycogen distribution in the liver with perivenous to periportal gradients was observed subsequently (not shown). Transplanted liver fragments did not show evidence of cholestasis, suggesting that bile was released in the blood followed by its clearance from the native liver.

Fate of Intraperitoneally Transplanted Liver Microfragments

Transplantation of liver microfragments in the peritoneal cavity was far less successful. Transplanted tissue failed to vascularize, and liver fragments progressively deteriorated, such that within 7 to 10 days, transplanted tissues showed considerable putrefaction. A progressive phagocytic response was initiated early, with extensive neutrophilic infiltration within 1 to 2 days and maximizing after 4 to 5 days following intraperitoneal implantation of liver fragments (not shown).

DISCUSSION

These studies establish that the small intestine can support an auxiliary liver. The major elements of the intestinal auxiliary liver were the use of isolated and viable intestinal segments, the removal of the intestinal mucosa to facilitate vascularization of the transplanted liver, and the use of tissue microfragments to facilitate mass transfer and revascularization. In contrast, the fate of liver microfragments transplanted in the peritoneal cavity was quite different, indicating that site-specific differences promoted survival of liver microfragments in the small intestine.

This heterotopic liver in the intestine is novel, although intestinal segments containing the submucosa have previously been used to develop arterial and venous grafts in animals (*18*). Efforts to generate vascular substitutes used a core of jejunal wall obtained by removing luminal layers containing the intestinal mucosa and lamina propria, in addition to abluminal serosal and tunica muscularis layers. These manipulations generated a tubular remnant of the jejunum, which withstood suturing to vessels for use as either arterial or venous grafts, for example, to bridge the portal vein and the inferior vena cava and as superior vena caval interposition grafts (*19*,*20*). Remarkably, such intestinal vascular grafts demonstrated remodeling, with the appearance of endothelial cell layers toward the luminal side containing blood. Moreover, in one study, hepatocytes were seeded in hollow fibers for placement in the portal bloodstream provided by an intestinal portacaval vascular graft (*19*). The authors believed that bathing of cells in portal blood would provide access to various hepatotropic factors. However, the description of the fate of hepatocytes in the intestinal vascular grafts was limited, and transplanted cell morphology or cell fate was not shown (*19*). Our system was obviously quite different from these manipulations. In contrast with vascular graft surgery, in which the intestinal segment was completely resected and denuded from both the mucosal and serosal sides, the vascular supply of our intestinal segments was maintained with removal of only the intestinal mucosa. Moreover, we implanted intact liver fragments in the intestinal segment without providing additional portal blood supply. Our concept was that use of the vascularized intestinal segment would provide opportunities for revascularization of transplanted tissue, leading to appropriate mass exchange.

Previous insights from transplantation of hepatocytes in the peritoneal cavity indicated that transplanted cell survival required various scaffolds or nonparenchymal liver cells (*16*,*21–23*). In our case, liver microfragments obviously contained nonparenchymal cells along with extracellular matrix components, and differences in the processes of revascularization in the peritoneal cavity versus the intestine should be an important consideration. Studies using large liver fragments were unsuccessful in establishing heterotopic auxiliary liver (reviewed in 17), whereas our use of smaller liver fragments likely facilitated oxygenation of transplanted tissues.

Remarkably, transplanted liver microfragments survived in the intestine despite the absence of portal blood flow, because intestinal segments were isolated from the remaining gut. In contrast, studies of auxiliary liver grafts suggested that portal blood supply is critical for regenerating the grafted liver (*12*,*13*). Whether the presence of various liver cell types within the microfragments or other unknown factors contributed to the production of hepatotropic growth factors, and promoted survival of liver tissue in the intestine, is another possibility. It may be relevant that liver and intestine share an embryologic origin from the foregut. Moreover, intestinal subepithelial myofibroblast (ISMF) cells, which were not removed by our procedures, play important roles in epithelial-mesenchymal cell interactions. For example, ISMF cells secrete multiple growth factors, including hepatocyte growth factor and transforming growth factor (TGF)-β (*24*). Hepatocyte growth factor promotes hepatocellular DNA synthesis in concert with additional hepatic growth factors and facilitates induction of differentiation in stem cells along the hepatocyte lineage, whereas TGF-β inhibits hepatic DNA synthesis (*25–27*).

Such interactions between growth-promoting and growth-inhibiting activities are critical for hepatic homeostasis in normal animals. We noted continuation of regulated liver growth in the intestinal segment because transplanted liver

fragments did not hypertrophy, which would have indicated loss of liver growth control. We did not observe mitotic activity or extensive apoptosis on microscopic examination, which was in agreement with the absence of significant hepatocellular turnover in transplanted liver tissues. However, our animals were subjected to autotransplantation, which required 35% hepatectomies, because we did not have access to syngeneic animals in our institution in the Republic of Georgia. Partial hepatectomy could potentially have facilitated engraftment of transplanted liver fragments after release of humorally active substances into the circulation. To discount this possibility, we are performing additional studies in syngeneic rats, in which autotransplantation is not needed.

The intestinal lining of the auxiliary liver showed fibrosis, which probably represented, at least in part, responses to surgical trauma and removal of the intestinal mucosa. Of course, excessive production or local availability of TGF-β from ISMF cells could have contributed toward generation of fibrogenic stimuli (*24*). Whether fibrosis in portal areas and bile duct proliferation in liver tissues in the intestinal segment were related to TGF-β–mediated mechanisms is a possibility, although additional mechanisms could be responsible; further investigation is required to define this process. One obvious way to test this hypothesis involves incorporation of specific growth factors or cytokines in our heterotopic liver transplant experiments.

We were impressed with the maintenance of liver plate structure and interspersed sinusoids lined with littoral cells in the intestine, which was similar to the normal liver. Moreover, the presence of glycogen in liver tissues was in agreement with the retention of expected hepatic function. These encouraging features of the intestinal auxiliary liver indicate that such a system will be relevant for addressing biological mechanisms and for clinical applications. The latter will concern metabolic deficiency states, acute liver failure, and chronic liver disease, such as Crigler-Najjar syndrome and other conditions (*8–11*). Whether the intestinal auxiliary liver will be successful in these situations can be tested in the Gunn rat model of Crigler-Najjar syndrome, in which correction of disease requires approximately 5% to 10% of the liver mass as indicated by hepatocyte transplantation studies (*28*).

Although liver mass in the single intestinal segment used in these studies was approximately 5%, we have been successful in isolating multiple intestinal segments in rats, with establishment of up to three discrete auxiliary livers approaching 15% to 20% of the liver mass (not shown). This type of manipulation should be particularly helpful in acute liver failure, in which one could envision combining hepatocyte transplantation and intestinal liver to assist recovery of the native liver. In chronic liver failure, additional masses of functionally intact liver should facilitate detoxification of accumulated ammonia and other toxins to resolve hepatic encephalopathy and hepatorenal syndrome (*21*,*29*). If the transplanted intestinal liver begins to reconstitute secretory proteins, for example, coagulation factors, this could help treat bleeding diatheses and even hemophilia (*30*). Although the extent of secretory function in the intestinal liver requires further study, the histologic integrity of the transplanted tissue and the absence of cholestasis indicate that such an auxiliary liver should be an excellent source of secretory proteins. In the current configuration, bile clearance will require the native liver, and the intestinal auxiliary liver will obviously be unsuitable for cholestatic biliary diseases.

REFERENCES

1. Jalan R, Williams R. Bio-artificial liver support for acute liver failure: should we be using it to treat patients? Transplantation 2002; 73165–73166.
2. Gagandeep S, Sokhi R, Slehria S, et al. Hepatocyte transplantation improves survival in mice with liver toxicity induced by hepatic overexpression of Mad1 transcription factor. Mol Ther 2000; 1: 358–365.
3. Bilir BM, Guinette D, Karrer F, et al. Hepatocyte transplantation in acute liver failure. Liver Transpl 2000; 6: 32–40.
4. Kayler LK, Merion RM, Lee S, et al. Long-term survival after liver transplantation in children with metabolic disorders. Pediatr Transplant 2002; 6: 295–300.
5. Durand F, Belghiti J, Handra-Luca A, et al. Auxiliary liver transplantation for fulminant hepatitis B: results from a series of six patients with special emphasis on regeneration and recurrence of hepatitis B. Liver Transpl 2002; 8: 701–707.
6. Jaeck D, Boudjema K, Audet M, et al. Auxiliary partial orthotopic liver transplantation (APOLT) in the treatment of acute liver failure. J Gastroenterol 2002; 37(suppl 13): 88–91.
7. Azoulay D, Samuel D, Ichai P, et al. Auxiliary partial orthotopic versus standard orthotopic whole liver transplantation for acute liver failure: a reappraisal from a single center by a case-control study. Ann Surg 2001; 234: 723–731.
8. Kiuchi T, Edamoto Y, Kaibori M, et al. Auxiliary liver transplantation for urea-cycle enzyme deficiencies: lessons from three cases. Transplant Proc 1999; 31: 528–529.
9. Rela M, Muiesan P, Vilca-Melendez H, et al. Auxiliary partial orthotopic liver transplantation for Crigler-Najjar syndrome type I. Ann Surg 1999; 229: 565–569.
10. Angelis M, Pegelow CH, Khan FA, et al. En bloc heterotopic auxiliary liver and bilateral renal transplant in a patient with homozygous protein C deficiency. J Pediatr 2001; 138: 120–122.
11. Haberal M, Arda IS, Karakayali H, et al. Successful heterotopic segmental liver transplantation from a live donor to a patient with Alagille syndrome. J Pediatr Surg 2001; 36: 667–671.
12. Kaibori M, Egawa H, Inomata Y, et al. Selective portal blood flow diversion in auxiliary partial orthotopic liver transplantation to induce regeneration of the graft. Transplantation 1998; 66: 935–937.
13. de Jonge J, Madern GC, Terpstra OT, et al. Directing portal flow is essential for graft survival in auxiliary partial heterotopic liver transplantation in the dog. J Pediatr Surg 1999; 34: 1265–1268.
14. Kusano M, Mito M. Observations on the fine structure of long-survived isolated hepatocytes inoculated into rat spleen. Gastroenterology 1982; 82: 616–628.
15. Demetriou AA, Whiting JF, Feldman D, et al. Replacement of liver function in rats by transplantation of microcarrier-attached hepatocytes. Science 1986; 233: 1190–1192.
16. Sundback CA, Vacanti JP. Alternatives to liver transplantation: from hepatocyte transplantation to tissue-engineered organs. Gastroenterology 2000; 118: 438–442.
17. Gupta S, Roy Chowdhury J. Hepatocyte transplantation: back to the future! Hepatology 1992; 15: 156–162.
18. Lantz GC, Badylak SF, Hiles MC, et al. Small intestinal submucosa as a vascular graft: a review. J Invest Surg 1993; 6: 297–310.
19. Kim SS, Kaihara S, Benvenuto MS, et al. Small intestinal submucosa as a small-caliber venous graft: a novel model for hepatocyte transplantation on synthetic biodegradable polymer scaffolds with direct access to the portal venous system. J Pediatr Surg 1999; 34: 124–128.
20. Robotin-Johnson MC, Swanson PE, Johnson DC, et al. An experimental model of small intestinal submucosa as a growing vascular graft. J Thorac Cardiovasc Surg 1998; 116: 805–811.
21. Umehara Y, Hakamada K, Seino K, et al. Improved survival and ammonia metabolism by intraperitoneal transplantation of microencapsulated hepatocytes in totally hepatectomized rats. Surgery 2001; 130: 513–220.
22. Benoist S, Sarkis R, Barbu V, et al. Survival and functions of encapsulated porcine hepatocytes after allotransplantation or xenotransplantation without immunosuppression. Surgery 2001; 129: 606–616.
23. Selden C, Calnan D, Morgan N, et al. Histidinemia in mice: a metabolic defect treated using a novel approach to hepatocellular transplantation. Hepatology 1995; 21: 1405–1412.
24. Powell DW, Mifflin RC, Valentich JD, et al. Myofibroblasts. II. Intestinal

subepithelial myofibroblasts. Am J Physiol 1999; 277: C183–C1201.
25. Tomiya T, Ogata I, Yamaoka M, et al. The mitogenic activity of hepatocyte growth factor on rat hepatocytes is dependent upon endogenous transforming growth factor-alpha. Am J Pathol 2000; 157: 1693–1701.
26. Miyazaki M, Akiyama I, Sakaguchi M, et al. Improved conditions to induce hepatocytes from rat bone marrow cells in culture. Biochem Biophys Res Commun 2002; 298: 24–30.
27. Yamano T, Hirai R, Hato S, et al. Delayed liver regeneration with negative regulation of hepatocyte growth factor and positive regulation of transforming growth factor-beta1 mRNA after portal branch ligation in biliary obstructed rats. Surgery 2002; 131: 163–171.
28. Guha C, Parashar B, Deb NJ, et al. Normal hepatocytes correct serum bilirubin after repopulation of Gunn rat liver subjected to irradiation/partial resection. Hepatology 2002; 36: 354–362.
29. Cai J, Ito M, Nagata H, et al. Treatment of liver failure in rats with end-stage cirrhosis by transplantation of immortalized hepatocytes. Hepatology 2002; 36: 386–394.
30. Gordon FH, Mistry PK, Sabin CA, et al. Outcome of orthotopic liver transplantation in patients with haemophilia. Gut 1998; 42: 744–749.

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DESPITE EFFICIENT INTRATHYMIC NEGATIVE SELECTION OF HOST-REACTIVE T CELLS, AUTOIMMUNE DISEASE MAY DEVELOP IN PORCINE THYMUS-GRAFTED ATHYMIC MICE: EVIDENCE FOR FAILURE OF REGULATORY MECHANISMS SUPPRESSING AUTOIMMUNITY[1]

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Background. **CD4 T-cell reconstitution and xenogeneic tolerance is achieved in T cell-depleted, thymectomized C57BL/6 (B6) mice and nude mice by grafting of fetal pig thymus (FP THY). Sixty percent of grafted nude mice and 10% of grafted thymectomized B6 mice develop a clinical illness resembling chronic graft-versus-host disease.**

Methods. **Negative selection of mouse T cells in FP THY grafts was studied in "AND" TCR transgenic mice with a negative selecting MHC. Pathologic and immunohistochemical examinations and adoptive transfer assays were performed to determine the role of mouse $CD4^+$ cells in the occurrence of autoimmune disease in this model.**

Results. **Marked clonal deletion of mouse thymocytes bearing a transgenic TCR ("AND"), which recognizes $H2^s$ expressed by host hematopoietic cells, was observed in FP THY grafts. Pathologic and immunohistochemical examinations of the liver, skin, lungs, and kidneys of mice with wasting syndrome showed marked mouse $CD4^+$ T-cell infiltration without detectable pig cells. After adoptive transfer of splenocytes, but not of $CD4^+$ cell–depleted splenocytes, from sick mice along with B6 bone marrow cells to lethally irradiated syngeneic B6 mice, the secondary recipients developed a similar autoimmune syndrome as the donors. Cotransfer of naïve syngeneic splenocytes prevented the occurrence of autoimmune disease in secondary recipients of splenocytes from healthy FP THY-grafted BALB/c nude mice.**

Conclusion. **These results demonstrate a key role for mouse $CD4^+$ T cells in causing autoimmune disease in this model and suggest the importance of regulatory mechanisms in addition to intrathymic clonal deletion for the maintenance of tolerance to recipient antigens.**

We recently demonstrated that donor-specific xenograft tolerance can be induced in thymectomized (ATX), T cell-depleted (TCD) mice by grafting with fetal pig thymus and liver tissue (FP THY/LIV) (*1–3*). In this pig-to-mouse model, functional mouse $CD4^+$ T cells repopulated the periphery of TCD ATX mice after grafting with FP THY/LIV (*4*). Repopulation did not occur in animals receiving similar treatment without FP THY grafts (*4*). The repopulated mouse $CD4^+$

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