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Rescue of Lethal Hepatic Failure by Hepatized Lymph Nodes in Mice

Toshitaka Hoppo¹, Junji Komori¹, Rohan Manohar¹, Donna Beer Stolz², and Eric Lagasse¹

¹ McGowan Institute for Regenerative Medicine, Department of Pathology University of Pittsburgh Medical School, Pittsburgh PA

² Cell Biology and Physiology, Center for Biologic Imaging University of Pittsburgh Medical School, Pittsburgh, PA

Abstract

BACKGROUND & AIMS—Hepatocyte transplantation is a potential therapeutic approach for liver disease. However, most patients with chronic hepatic damage have cirrhosis and fibrosis, which limit the potential for cell-based therapy of the liver. The development of an ectopic liver as an additional site of hepatic function represents a new approach for patients with an end-stage liver disease. We investigated the development and function of liver tissue in lymph nodes in mice with liver failure.

METHODS—Hepatocytes were isolated from 8 to 12-week-old mice and transplanted by intraperitoneal injection into 8- to 12-week-old *Fah*^{-/-} mice, a model of the human liver disease tyrosinemia type I. Survival was monitored and the locations and functions of the engrafted liver cells were determined.

RESULTS—Lymph nodes of *Fah*^{-/-} mice were colonized by transplanted hepatocytes; *Fah*⁺ hepatocytes were detected adjacent to the CD45⁺ lymphoid cells of the lymphatic system. Ten weeks after transplantation, these mice had substantial improvements in serum levels of transaminases, bilirubin, and amino acids. Homeostatic expansion of donor hepatocytes in lymph nodes rescued the mice from lethal hepatic failure.

CONCLUSIONS—Functional ectopic liver tissue in lymph nodes rescues mice from lethal hepatic disease; lymph nodes might therefore be used as sites for hepatocyte transplantation.

Keywords

liver transplantation; liver regeneration; ectopic organogenesis; cell therapies

Orthotopic liver transplantation (OLT) is currently the only curative treatment for severe liver disease. However, due to the shortage of donor organs, its application is greatly limited. Furthermore, patients with co-morbidities and advanced age are either not

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Correspondence: Eric Lagasse, Pharm. D, Ph.D. University of Pittsburgh McGowan Institute for Regenerative Medicine 450 Technology Drive, Suite 300 Pittsburgh, PA 15219 Phone: 412-624-5285 Fax: 412-624-5263 Lagasse@Pitt.edu.

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considered candidates for OLT or are expected to have reduced post-transplant survival¹⁻³. Cell-based transplantation has been proposed as a therapeutic alternative to OLT or as a bridge for patients who are waiting for an organ to become available⁴⁻⁶. Most cellular therapies for liver diseases have been directed at cell engraftment in the liver itself. This approach limits the possible efficacy of cellular therapy in the vast majority of patients with end-stage liver diseases where cirrhosis and fibrosis are the common pathological features⁷⁻⁹. The development of an ectopic liver as an additional site of hepatic function represents a new therapeutic opportunity for patients with an end stage liver disease who would be at high risk for OLT. Transplantation of hepatocytes at several different extra-hepatic sites has been demonstrated in animal models, but engraftment of hepatocytes has been associated with variable results and no study has demonstrated that the extra-hepatic tissue mass could rescue the function of the liver itself^{10, 11}. Here we demonstrate that the development of life-supporting ectopic liver tissue is possible in lymph nodes after liver failure.

Materials and Methods

Animals

Fah^{-/-} mice (129sv) kindly gifted by Dr. Markus Grompe (Portland, OR) or Fah^{-/-} mice backcrossed into C57bl were used for recipients and 129S4 and GFP-C57Bl mice (Cat#004353) obtained from The Jackson Laboratory (Bar Harbor, ME) were used for donors. Freshly isolated hepatocytes were obtained from 8 to 12-week-old mice and were transplanted into 8- to 12-week-old Fah^{-/-} mice. The protocol followed National Institutes of Health guidelines for animal care and was approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Cell preparation

Hepatocytes were harvested using the 2-step collagenase perfusion technique introduced by Seglen¹². The number and viability of cells were determined by trypan blue exclusion. One million viable cells were suspended in 30μl HBSS and kept on ice until transplantation.

Transplantation

For intraperitoneal hepatocyte transplantation, one million viable liver cells were injected into the lower peritoneal cavity with a 28-gauge needle. For splenic hepatocyte transplantation, animals were anesthetized and a small surgical incision was made in the left flank. The spleen was exposed and 0.2×10^6 liver cells, suspended in 30μl HBSS, were injected into the inferior pole of the spleen using a 28-gauge needle. The injection site was ligated to prevent cell leakage and bleeding. All mutant mice were kept on 2-(2-nitro-4-trifluoro-methylbenzyl)-1,3-cyclohexanedione (NTBC) until transplantation. NTBC was discontinued just after transplantation. The weight of experimental animals was taken weekly to monitor their health. Generally, Fah^{-/-} mice lose weight during the first few weeks after transplantation due to the gradual loss of liver function and progressively regain their initial weight later when donor liver cells regenerate liver tissue and hepatic functions. Whenever the animals lost more than 25% of their initial body weight, the risk of losing these animals increased and NTBC is given back to restore liver function. It usually took 5-7 days for the mice to return to the initial weight and liver functions under NTBC. At that point, NTBC is discontinued again to induce liver failure. Such protocol was used previously to allow a low number of engrafting liver cells to selectively generate enough liver mass to finally rescue the animals from liver failure¹³.

Fah enzyme assay

Fah enzyme assays were carried out at 37°C as described previously¹⁴. The harvested tissues stored at -80°C were homogenized and sonicated in complete lysis M buffer (Roche, Mannheim, Germany). Protein concentrations were measured with BCA protein assay kit (Pierce, Rockford, IL) and adjusted to 3µg/ml. 8µl of fumarylacetoacetate (FAA, a gift from Dr. Grompe), the substrate for this assay, was incubated with each protein solution and the attenuation of absorbance at 330nm was measured spectroscopically every 10sec. Wt and Fah^{-/-} livers were used as positive and negative controls. FAA is not commercially available and was prepared enzymatically from homogentisic acid¹⁴.

Serial transplantation of hepatocytes from hepatized lymph nodes

Harvested hepatized lymph nodes were minced into small pieces and incubated in 0.1mg/ml collagenase type II solution supplemented with 0.05mg/ml DNase I (Sigma) at 37°C for 30min. The isolated cells were collected by filtration through a 70µm nylon mesh and washed three times with HBSS. The number and viability of cells were determined by trypan blue exclusion. 10⁵ cells were suspended in 30µl HBSS and transplanted by splenic injection as described above. The repopulation of Fah positive hepatocytes in the recipient liver was calculated by counting the number of Fah positive cells in any four views randomly selected on Fah stained sections.

Results

Intraperitoneal injection of hepatocytes rescues mice from liver failure

We transplanted Fah^{-/-} mice to explore the feasibility of functional ectopic liver in a model of highly efficient liver regeneration^{15, 16}. Fah^{-/-} tyrosinemic mice have progressive and fatal liver failure unless treated with 2-(2-nitro-4-trifluoro-methylbenzyl)-1,3-cyclohexanedione (NTBC, nitisone, Orphadin®)¹⁵. We and others have shown that wild type (wt) hepatocytes have a strong selective growth advantage when transplanted in the liver of Fah^{-/-} mice after NTBC removal, resulting in near-complete regeneration of the liver^{13, 16}. To evaluate a possible ectopic location for liver cell transplant, 10⁶ liver cells from congenic wt mice were transplanted in Fah^{-/-} tyrosinemic mice intraperitoneally (IP) (n=50). Splenic injection (SP) was used as a positive control, indirectly delivering the cells to the liver^{17, 18} (n=21). NTBC was removed to induce progressive liver failure in all the transplanted animals and their weight was monitored weekly as an indicator of liver function. SP injected mice initially lost weight and then spontaneously regained weight (Figure 1A) with donor hepatocytes repopulating the entire diseased liver and reversing lethal tyrosinemia, as described previously¹⁶ (19/21 mice transplanted, 90.4% survival). IP transplantation of liver cells resulted in long-term survival of these animals (Figure 1A). Long-term survival was successful with one period of selection (4/11 mice transplanted, 36% survival) but with less efficiency than after two periods of selection (42/50 mice transplanted, 84% survival) (Figure 1B).

Ten weeks post transplantation and after apparent rescue of tyrosinemia, laparotomies were performed on the experimental mice. Twenty to forty enlarged nodules were observed around the stomach region and along the mesentrium in all the IP transplanted animals (Figure 1C). None of these enlarged nodules were found in SP transplanted Fah^{-/-} mice. The distribution of nodules matched the expected distribution of lymph nodes present in these regions. The presence of enlarged nodules and the reversal of tyrosinemia was a long-lasting effect. Over six months after transplantation, Fah^{-/-} animals were still alive and healthy.

Lymph nodes are colonized by hepatocytes

At various times after IP transplantation of hepatocytes, *Fah*^{-/-} mice were sacrificed to determine the origin of the hepatic nodules. Within few days, *Fah*⁺/*CK18*⁺ hepatocytes were detected adjacent to the *CD45*⁺ lymphoid cells of the lymphatic system. *CK18*⁺ hepatocytes co-localized with *Meca79* (*CD62L* ligand), a marker of high endothelial venules (HEVs) present in lymph nodes (Figure 2A). Several weeks after transplantation, *Fah*⁺ hepatocytes had entirely colonized several lymph nodes (Figure 2B and 3). Furthermore, BrdU labeling experiments indicated that donor hepatocytes in the lymph nodes proliferate for 2-3 weeks after transplantation and cease to proliferate by 8 weeks post-injection (Figure 2B). Immunofluorescent analyses of hematopoietic markers (*CD45*) with T-cell markers (*CD3*, *CD4* and *CD8*), B-cell marker (*B220*) and myeloid markers (*Gr-1* and *CD11b*) suggest the transformation of the lymph nodes from a lymphoid organ to an hepatic organ with the presence of *CK18*⁺ hepatocytes (Figure 2C) ten weeks after transplantation. Using GFP labeled donor liver cells, we found that no other organs were colonized except visceral lymph nodes and occasionally native liver with small colonies. This result suggests that hepatocytes rapidly migrate into the lymphatic system through afferent lymph vessels, colonize lymph nodes, proliferate, and then passively or actively eliminate lymphocytes from the lymph nodes.

Characterization of hepatized lymph nodes

Immunohistological analyses confirmed the presence of donor hepatocytes in all the analyzed enlarged nodules (Figure 3A and 3B). The newly generated hepatized lymph nodes had a hepatic mass representing over 70% of expected normal liver mass or 1.5×10^7 liver cells (Figure 3C). This massive ectopic engraftment and expansion of liver cells subsequently rescued the animal from lethal liver failure. Analysis of the hepatized lymph nodes showed that not only had the lymphocytes almost completely disappeared (Figure 2C), but HEVs, the specialized postcapillary venules found in lymphoid tissue¹⁹, were also absent following hepatocyte colonization (Figure 3B). The HEVs were replaced with large vessels that have a histology and size similar to that found in normal liver (Figure 3B), but lacked the characteristic fenestrations (Figure 3D). These vessels appeared to be abundant in hepatized lymph nodes indicating a possible adaptation of the vasculature to the newly generated hepatic tissue.

On the other hand, ER-TR7, a marker for reticular fibroblasts and reticular fibers, was missing in the hepatized lymph nodes, as well as F4/80, a marker for macrophage/Kupffer cells (Figure 3E). *CK19*, a marker for biliary epithelial cells, was also absent but the presence of bile canaliculi around desmosomes and tight junctions between hepatocytes was confirmed by electron microscopy (Figure 3D). *Desmin* and *GFAP*, two markers of stellate cells, were detected. However, because these markers were also present in normal lymph nodes, definitive identification of the donor's hepatic stellate cells was inconclusive (Figure 3E).

Biochemical liver functions are restored by hepatized lymph nodes

We assessed the biochemical liver function of *Fah*^{-/-} mice transplanted IP or SP, normal wt donor mice, *Fah*^{-/-} mice under NTBC and untreated *Fah*^{-/-} mice (under liver failure) by measuring serum levels of transaminases, bilirubin and amino acids (Figure 4A). Ten weeks after transplantation, IP injected *Fah*^{-/-} mice showed substantial improvement in all parameters. They differed from SP mice by a slight decrease in some of the liver functions. Interestingly, concentrations of both total and direct bilirubin were abnormally elevated in the serum of IP mice, but were several folds lower than untreated tyrosinemic mice in hepatic failure (Figure 4A). The higher concentration of bilirubin is explained by the absence

of CK19⁺ biliary cells observed in the hepatized lymph nodes (Figure 3E), even though biliary canaliculi containing bile were present between hepatocytes (Figure 3D).

Furthermore, the levels of serum albumin, fibrinogen and HGF as well as BUN, total cholesterol and triglyceride were analyzed in the IP injected Fah^{-/-} mice and compared to normal mice (Figure 4B and C). Serum albumin and fibrinogen plasma levels were restored to normal levels. Interestingly, serum HGF shows an increase at 6 weeks after transplantation. This increase in serum HGF correlates with the massive expansion of hepatocytes expected in lymph nodes around 6 weeks after transplantation. Serum BUN was normal but total cholesterol was slightly increased and triglyceride was significantly lower than wild type normal mice. The slight discrepancy in the lipid analyses found in the IP transplanted animals may be explained by the variability observed between normal male and female mice. The transplantations were not sex-matched which could explain some differences observed in the experimental animals. Glycogen storage was determined by Periodic Acid Schiff (PAS) staining of hepatized lymph node in liver sections, and appeared normal. In addition, electron microscopy was used to identify the glycogen rosettes in a lymph node derived hepatocyte (Figure 4D).

Analyses of Fah activity in hepatized lymph nodes and native liver

Occasionally, small intra-hepatic nodules of donor hepatocytes were identified in native tyrosinemic livers of IP injected mice (Figure 1C). We hypothesized that hepatocytes might have drained from the lymphatic system into the bloodstream via the subclavian vein and subsequently, into the liver. Fah enzyme activity was measured¹⁴ in order to estimate the number of donor hepatocytes in the native liver versus hepatized lymph nodes, and more importantly, to determine if they contribute to the restoration of liver function (Figure 4E). Fah enzyme activities in hepatized lymph nodes ranged from 80% to almost 100% of wt liver levels. In contrast, Fah enzyme activities in native tyrosinemic liver had a mean activity close to 15% of wt liver levels. There was a complete lack of Fah enzyme activity in the native tyrosinemic liver of 1 out of 5 mice, which is comparable to untreated Fah^{-/-} mice. No significant correlation was found between the level of serum bilirubin (an indicator of liver function) and the Fah activity level in native livers of the transplanted Fah^{-/-} mice (an indicator of wild type hepatocytes engrafted in the liver)(Supplementary Figure 1). These results indicate that the presence of wild type hepatocytes sometimes found in the liver of the Fah mice could not explain the massive expansion of liver cells in lymph nodes and restoration of liver functions observed with the survival of the animals.

Expansion of the hepatized lymph nodes after partial hepatectomy

Partial hepatectomy is known to lead to regeneration of the remnant liver²⁰. Here we asked whether hepatized lymph nodes would respond to the same regenerative triggers after partial hepatectomy. Fah^{-/-} mice were transplanted IP with 10⁶ wt hepatocytes. Ten weeks after hepatic engraftment in lymph nodes, a partial hepatectomy was performed in 3 of the 6 transplanted Fah^{-/-} mice. Hepatic regeneration was induced by surgically removing the median and left lateral hepatic lobes, representing two-thirds of the liver mass. Three weeks later, all 6 mice were sacrificed and their livers and hepatized lymph nodes were harvested. Partial hepatectomy showed that further resection of the native liver results in an expansion of the hepatized lymph nodes with survival of the animal (Figure 5). This result provides additional evidence that hepatized lymphnodes are responding to homeostatic mechanisms regulating the maintenance of liver tissue mass and liver function after injury.

Hepatocytes derived from hepatized lymph nodes are not tumorigenic

Hepatocyte migration and invasion into the lymph nodes represent a profound change in the morphology and behavior of epithelial cells reminiscent of the metastatic process. However,

such profound changes in behavior of epithelial cells are not always correlated with tumor progression and have been observed during embryonic development ²¹. Even in adult life, lymph nodes can contain benign inclusions of epithelial cells without malignant disease. To rule out malignant transformation, hepatocytes were isolated from hepatized lymph nodes and retransplanted via splenic injection into secondary *Fah*^{-/-} recipients (n=5). Three to six months after transplantation, none of the rescued animals showed the presence of either hepatocytes in the lymphatic system or tumors. The mean of hepatocyte repopulation in the liver was 85.3%, indicating the similar transplantability and therapeutic effect of lymph node derived hepatocytes when compared to liver derived hepatocytes (Figure 6).

Conclusions

Organ transplantation is too often the last resort for patients suffering from terminal disease. It is thought that tissue engineering and regenerative medicine have the potential to solve some of the problems associated with organ transplantation. Although the liver is an extraordinary organ due to its regenerative properties, engineered liver organogenesis is not yet a viable therapeutic option. Our goal was to identify an *in vivo* location where ectopic liver organogenesis would be feasible. In this study, we show that hepatocytes survive in lymph nodes and generate functional hepatized lymph nodes in an animal model of Type I tyrosinemia. Transplantation of liver cells in the peritoneal cavity allowed the hepatocytes to migrate into the lymphatic system, enter the lymph nodes, and expand under homeostatic mechanisms driven by the liver at the expense of lymph node lymphocytes. When ectopic liver tissue reached the required balance for hepatic function, proliferation ceased, resulting in twenty to forty hepatized lymph nodes that represented 70% of the original liver mass. In addition, we did not observe possible complications such as ascites or lower extremity edema expected by the intra-abdominal lymphadenopathy. Partial hepatectomy of the diseased liver further expanded the mass of hepatized lymph nodes, indicating the tight homeostatic control of liver mass has been retained at the ectopic site.

We speculate that the highly vascularized nature of the lymph nodes support the efficient engraftment and massive expansion of the ectopic tissue. It has been proposed that an inadequate vascular supply leads to hepatocyte death due to hypoxia within a few days of ectopic transplantation ^{10, 22}. Since lymph nodes are intended for the support of lymphocyte proliferation and expansion ²³, they may be better suited to the immediate survival of engrafting hepatocytes. Therefore lymph nodes could be compared to a well-designed “*in vivo* bioreactor” originally built for the rapid expansion of lymphocytes but now retasked for colonization by hepatocytes. While major advances have been made in scaffold and cell technology, the vascularization and nourishment of large tissues remains an engineering problem that could be resolved when hepatocytes engraft in lymph nodes.

The mechanism of expansion of hepatocytes in lymph nodes of *Fah*^{-/-} mice appeared similar to the very complex phenomenon of the regenerative process following partial hepatectomy. In this process, well-orchestrated signaling cascades, characterized over many years, direct the restoration of the lost hepatic mass²⁰. However, in our experiments it remains unclear how hepatocytes or subpopulations of hepatocytes enter the lymph nodes. The mechanism of active or passive infiltration, neutralization of lymphocytes and reorganization of the architecture of the nodes to mimic functional hepatic tissue remains to be determined. We do know that the expansion of hepatocytes in lymph nodes is associated with essential factors of hepatic growth such as HGF. We have detected HGF during the development of ectopic liver tissue in the lymph nodes at 6 weeks post transplant, when the massive expansion of liver cells in lymph nodes rescues animal survival. We postulate that hepatocytes in the lymph nodes are susceptible to a mitotic stimulus much like hepatocytes after partial hepatectomy, responding to similar growth factors and expressing similar transcriptional

cascades, resulting in the restoration of liver homeostasis. Additional mechanisms for hepatocyte invasion and engraftment in the lymph nodes remain to be determined.

Although biliary canaliculi with microvilli could be observed on transmission electron microscopy, biliary morphogenesis revealed by CK 19 staining could not be detected in hepatized lymph nodes. Surprisingly, IP injection of hepatocytes resulted in a decrease in conjugated bilirubin, but not to normal levels. Our proposed mechanism for the improved hyperbilirubinemia, is the excretion of conjugated bilirubin by the diseased liver. This hypothesis is based on several observations. The presence of bile in the gallbladder of the animals indicates excretion by the native tyrosinemic liver (Supplementary Figure 1). The histology of the native tyrosinemic liver after rescue by IP injection supports these findings; the biliary cells appear normal and preserved while hepatocytes have abnormal morphology. Finally, we show no significant correlation between serum bilirubin levels and some of the low engraftment of the donor hepatocytes in the native livers, indicating that low levels of wild type hepatocytes sometimes found in the liver of the Fah mice could not explain the improved hyperbilirubinemia (Supplementary Figure 1). All these observations indicate that the ectopic liver tissue complements some of the functions of the native liver, which results in the improvement in hyperbilirubinemia observed in IP injected mice. Alternatively, conjugated bilirubin and bile salts could be excreted by the kidneys, especially in view of the very high glomerular filtration rate in mice, compared with that in other laboratory animals or humans.

Furthermore, both ER-TR7, a marker for connective tissue fibers, and F4/80, a marker for Kupffer cells were absent in the hepatized lymph nodes, but present in a normal liver. These results indicate that the architecture and cellular content of the ectopic liver tissues in lymph nodes differ from normal liver. However, hepatized lymph nodes will normalize most of the liver functions, including albumin, fibronectin, urea and lipid metabolism and most importantly, provide liver functions necessary for long-term survival of the tyrosinemic mouse.

Patients with end stage chronic liver disease have progressive hepatic failure that precludes any possible repair by the native liver. Liver disease in the Fah^{-/-} model also results in a progressive hepatic failure, preventing any possible repair by the native liver. Only long-term survival of functional donor hepatic tissue can contribute to survival of the Fah^{-/-} mouse. Our successful approach of generating functional ectopic liver tissue suggests that it may be possible one day to apply this methodology in patients suffering from liver diseases when liver transplantation or regeneration of the liver by cell-based therapy are not possible. Targeting lymph nodes for liver cell transplantation could be an approach to limit growth of hepatocytes to specific lymphatic sites. However, it still needs to be demonstrated that human hepatic insufficiency, in general, or in particular conditions like cirrhosis, can lead to a selective advantage for the transplanted liver cells. Although conditioning protocols for hepatocyte repopulation after liver cell transplantation have been reported by other investigators 10, 24-26, no effective protocol has been established and it is difficult to use any conditioning protocols reported for clinical application at present. Immunologic barriers may limit allogenic transplantation of hepatocytes. Allogenic hepatocytes, in the context of lymphatic tissue, should be investigated for the treatment of human hepatic failure. Recently, induced pluripotent stem (iPS) cells have been established from fibroblasts and other mature somatic cells, indicating that immunologic issues affecting allogenic transplantation may be circumvented in the future 27, 28. In conclusion, the therapeutic efficacy of hepatized lymph nodes in restoring liver function may represent a unique opportunity to treat certain patients with end-stage liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Writing Assistance

Toshitaka Hoppo:

Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript.

Junji Komori

Acquisition of data; analysis and interpretation of data.

Rohan Manohar

Acquisition of data; analysis and interpretation of data; critical revision of the manuscript; statistical analysis.

Donna Beer Stolz

Acquisition of data; analysis and interpretation of data.

Eric Lagasse

Study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; obtained funding; technical, or material support; study supervision.

Abbreviations

ALT	alanine aminotransferase
FAA	fumarylacetoacetate
Fah	fumarylacetoacetate hydrolase
FITC	fluorescein isothiocyanate
GFP	Green Fluorescent Protein
IP	Intraperitoneally
IVC	inferior vena cava
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexane dione

OLT	orthotopic liver transplantation
PE	phycoerythrin
SP	splenic
WT	wild type
BUN	blood urea nitrogen
HEVs	high endothelial venules

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Figure 1.

Fah^{-/-} mice are rescued from lethal hepatic failure by intraperitoneal injection of hepatocytes. (A) Body weight after splenic (SP) and intraperitoneal (IP) transplantation indicates hepatic regeneration. Body weight of the transplanted mice was monitored weekly after liver cell transplantation (time 0), in order to follow hepatic engraftment and rescue from tyrosinemia. *Fah*^{-/-} mice transplanted by either single SP or IP injections lost weight during the first 3 weeks. Weight loss is indicative of a decline in liver function. SP injected mice spontaneously regained weight. IP injected mice required two periods of selection prior to regaining weight for efficient survival, a protocol previously described for engrafting low levels of liver cells¹³. (B) Spontaneous weight gain after a single period of selection was possible but with a lower survival rate. *Fah*^{-/-} mice were transplanted with 10⁶ wt liver cells followed by NTBC removal from their diet (blue and red line). n = number of mice analyzed. (C) Anatomic location of enlarged nodules 10 weeks after transplantation. Left panel: many enlarged nodules around the stomach region and on the mesenterium are observed in a mouse transplanted IP with wt hepatocytes (circles). Middle upper panel: native liver of the IP injected *Fah*^{-/-} mouse and a control wt liver. The native liver of the IP injected *Fah*^{-/-} mouse was atrophic with a couple of small regenerative nodules containing wt hepatocytes on its surface (arrows). Middle lower panel: isolated enlarged nodules from mouse in the left panel with diameters from 1 to 10 mm. Right upper and lower panels: mesenteric lymph nodes (mln) repopulated with GFP positive liver cells. Blood vessels (bv) and small intestine (si) are GFP negative.

Figure 2.

Immunohistochemistry of lymph nodes from the gastric and common hepatic arteries. (A) 2 and 3 days after IP injection of hepatocytes in *Fah*^{-/-} mice. On day 2, some wt *Fah*⁺CK18⁺ hepatocytes could be detected in lymphatic system near lymphocytes. On day 3, clusters of CK18⁺ hepatocytes were seen in association with CD45⁺ hematopoietic cells. Meca79 (CD62L ligand), a marker of high endothelial venules found in lymph nodes, is co-localized with CK18⁺ donor hepatocytes (B) 2 and 3 weeks after IP injection, *Fah*⁺ hepatocytes (green) have colonized the lymph nodes and have a high index of proliferation, as demonstrated by the high ratio of BrdU incorporation (red nuclei). 8 weeks after IP injection, few liver cells are proliferating in lymph nodes. (C) Immunofluorescence analysis of hematopoietic markers in hepatized lymph nodes 10 weeks after IP injection of hepatocytes in *Fah*^{-/-} mice. Both sections of hepatized lymph nodes and control (wt) lymph nodes were stained with hematopoietic markers. Each staining has two panels, the upper panel represents the lymph nodes engrafted with hepatocytes (hepatized LN) and lower panel is normal wt mouse lymph node (Control LN). Stainings were performed on serial sections. Bar: 100μm.

Figure 3.

Hepatized lymph nodes 10 weeks after IP transplantation. (A) Sections were immunostained with an anti-Fah antibody (brown, HRP staining) then counterstained with hematoxylin. Fah⁺ hepatocytes and several islands of small hematopoietic cells were present but no biliary structures were observed. Bar: 100μm. (B) Immunofluorescence of lymph nodes engrafted with hepatocytes (hepatized LN), control liver and control lymph nodes. Frozen sections were stained with hepatocyte marker CK18 and the endothelial marker CD31. CD26, dipeptidyl-peptidase-IV, was used as a hepatocyte maker and E-Cadherin as an epithelial maker. Most cells in the hepatized LN were CK18⁺ hepatocytes, with expression patterns similar to those of control liver. These cells were also albumin positive (brown cells, insert in left upper panel) with CK18 and CD26 co-localized (insert in CD26 staining panel). Hepatocyte and epithelial markers were negative in normal lymph node. In the hepatized LN, CD31⁺ endothelial cells corresponding to vessels and were similar in size and morphology to those found in normal (control) liver. In contrast, CD31⁺ cells indicative of high endothelial vessels (HEV) found in normal (control) lymph nodes differ in morphology. Bar: 100μm. (C) The ratio $\pm$ s.d. of the weight of liver and enlarged nodules to body weight. The ratio of hepatic tissues to body weight was determined in Fah^{-/-} mice transplanted IP and both liver (atrophic) and enlarged nodules (hypertrophic) were collected and compared to normal wt liver. n = number of mice analyzed. (D) Transmission electron microscopy of the hepatized lymph nodes. Ultrastructure of a hepatized lymph node (upper panels) and control liver (lower panels). Left upper panel: hepatocytes present in lymph nodes have large prominent nuclei (N), bile canaliculi (BC), mitochondria (M), peroxisomes (P) and rough endoplasmic reticulum (RER). Bar: 2μm. Center upper panel: higher magnification of the bile canaliculus, containing microvilli (MV) with tight junctions (arrowheads) and adherent junctions (AJ). A lipid vacuole is seen within the canaliculus. Bar: 500nm. Right upper panel: Vessels in hepatized lymph node consisted of non-fenestrated sinusoidal endothelial cells (SECs). Bar: 1μm. Left lower panel: hepatocytes in

control liver showing fenestrations (arrows) in SECs. Bar: 2 μ m. Center lower panel: higher magnification of bile canaliculus showing tight junctions (arrowheads), lipid vacuoles and Space of Disse (SD). Bar: 500nm. Right lower panel: Organization of hepatic plates in control livers with bile canaliculi at the apical surface and fenestrated sinusoids (S) at the basolateral surface. Bar: 2 μ m. (E) Immunofluorescence analysis with non-hematopoietic liver cells markers in hepatized lymph nodes, normal liver and normal lymph node (LN). Staining was performed with the hepatocyte marker, CK18 and the non-parenchymal cell markers F4/80, Desmin, and GFAP, CK19 and ER-TR7. F4/80⁺ (Kupffer cells), CK19 (biliary cells), and ER-TR7 (reticular fibroblasts) were negative in hepatized lymph nodes. Bar: 100 μ m.

by ELISA. Each open circle represents the value from one mouse. *t*-tests were run using Prism to determine significant differences between particular groups. HGF increased at 6 weeks after the second and final selection (off NTBC) reflecting the massive expansion of hepatocytes in lymph nodes necessary for the *Fah*^{-/-} survival. Bars indicate mean values. For HGF * $p_{control \& 6wk} = 0.0079$ by Mann Whitney test. (C) Serum concentration of blood urea nitrogen (BUN), total cholesterol and triglycerides in *Fah*^{-/-} mice over 10 weeks after IP injection and rescue of the animals, and compared wild type mice with *p* values. (D) Glycogen storage in hepatized lymph nodes. Glycogen storage was determined by Periodic Acid Schiff (PAS) staining. Bar: 100 μ m. The black and white electron microscopy panel identified glycogen rosettes (arrowheads) in hepatocytes. Bar: 500nm. (E) *Fah* enzyme assay. A standard curve to measure enzyme activity was established using wt liver (100% activity), *Fah*^{-/-} liver (0% activity) and wt/*Fah*^{-/-} mixes to achieve 15%, 25% and 80% enzyme activity. *Fah* enzyme activities in engrafted lymph nodes (LN) ranged from 80% to almost 100% of wt liver levels. In contrast, *Fah* enzyme activity in native livers of *Fah*^{-/-} mice rescued by hepatized LN had *Fah* activity ranging from 25% of wt liver activity to 0% (mean *Fah* activity of 15% of wt liver levels). n = number of mice analyzed.

Figure 5.

Expansion of hepatized lymph nodes after hepatectomy. (A) Anatomic location of hepatized lymph nodes in IP injected *Fah*^{-/-} mice after hepatectomy. Enlarged nodules were found around the stomach region and on the mesenterium (yellow circles). (B) Native liver and extra-hepatic nodules after hepatectomy from the mouse on the left panel. The native tyrosinemic liver of the IP injected *Fah*^{-/-} mouse was atrophic and the enlarged nodules had a diameter from 3 to 15 mm. (C) The ratio $\pm$ sem of the weight of native liver and hepatized lymph nodes to body weight. The ratio of hepatic tissues to body weight was determined between transplanted *Fah*^{-/-} mice with or without hepatectomy. The hepatized lymph nodes

show a significant increase in their weight after hepatectomy ($P=0.0052$). n = number of mice analyzed.

Figure 6.

Serial transplantation of lymph node derived hepatocytes. Hepatocytes isolated from hepatized lymph nodes were serially transplanted into *Fah*^{-/-} mice by splenic (SP) injection. (A) Body weight of *Fah*^{-/-} mice after splenic transplantation. The body weight lost and spontaneous gain after lymph nodes derived hepatocytes transplantation is very similar to the change observed when liver derived hepatocytes are transplanted. Two selections were necessary due to the low number of hepatocytes transplanted. (B) *Fah*⁺ hepatocytes were observed only in the repopulated liver of *Fah*^{-/-} mice 8 weeks after transplantation. Counterstaining was done with eosin. Bar: 100μm.