Tissue Distribution of Fetal Liver Cells Following *In Utero* Transplantation in Mice

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Transplantation of hepatic stem cells *in utero* has been advanced as a potential clinical approach to a variety of diseases, including deficiencies of coagulation factors. Although syngeneic transplantation has met with some success, consideration needs to be given to the potential for transplanted cells to colonize nontarget tissues. Liver cells were harvested from Rosa26 embyros at embryonic age 12.5 days postconception (pc) and transplanted into the peritoneal cavity of syngeneic recipients *in utero*. Tissues were harvested from tissue recipients at various time points ranging from 1 to 328 days pc, and tissues were stained for β-galactosidase to identify the existence of cells derived from Rosa26 donors. β-galactosidase-positive cells were found in the lung, liver, and brain as early as 20 days pc and through 328 days pc. Positive cells in these tissues existed as islands of cells that were morphologically similar to hepatocytes. In the spleen, individual β-galactosidase-positive cells of both leukocytic and erythrocytic lineages were present, and suggest that hematopoietic cells were transferred to recipients along with hepatocytes. The lack of an inflammatory response to the β-galactosidase-positive cells suggests that the donor cells were immunologically tolerated. In summary, the possibility that cells administered *in utero* may inadvertently colonize nontarget tissues suggests that clinical application of this method will need to be approached with diligence. Exp Biol Med 230:860–864, 2005.

Key words: hepatocytes; stem cells; transplantation; β-galactosidase

Introduction

Maintaining the hemostatic balance between coagulation and anticoagulant factors is necessary to sustain the proper flow of blood through the vasculature. Insufficient expression of factors such as FII, FV, FVII, FVIII, FIX, FX, and FXI result in hemorrhagic disorders. Because many hemostatic factors are primarily synthesized in the liver, it is useful to consider impaired expression of these factors as a hepatic dysfunction; thus, therapeutic strategies to treat hemophilia A or B include increasing hepatic expression of FVIII or FIX, respectively (1).

Bleeding diatheses resulting from FVII deficiencies have recently been treated by orthotopic liver transplantation (2). However, the procedure is complex and highly invasive. Combined with the relative paucity of donor organs, liver transplantation is an approach unlikely to be of widespread practical utility.

An alternative therapeutic approach might be the implantation of hepatocyte progenitors from a donor with normal hepatic function. In mouse and rat models, the ability of donor hepatocytes to colonize the liver following chemical-induced injury has been demonstrated (3–13). None of these studies specifically demonstrated the ability of transplanted hepatocytes to restore impaired blood clotting.

We recently showed that FX-deficient mice could be rescued from a bleeding diathesis by *in utero* implantation of liver cells from fetal wild-type mice (14). In that report, we observed that foci of donor cells had colonized the recipient liver. Because fetal livers contain pluripotent stem cells, the study described here was undertaken to evaluate the ability of transplanted fetal liver cells to colonize other recipient tissues.

Materials and Methods

Animals. Studies used Rosa26 (15) mice that were bred to generate fetuses for liver cell harvest and wild-type 129/C57J mice (The Jackson Laboratory, Bar Harbor, ME), which were bred to generate fetuses to be used as fetal liver cell recipients. Mice were housed in polycarbonate cages with chopped corn cob bedding and were provided *ad libitum* access to fresh water and food (Purina Rodent Chow; Purina, St. Louis, MO). Animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and

This work was supported by the National Heart, Blood, and Lung Institute through grants HL060081 to E.D.R. and PPG-HL-073750 to F.J.C. and E.D.R.

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Received June 21, 2005.
Accepted August 18, 2005.

1535-3702/05/23011-0860$15.00

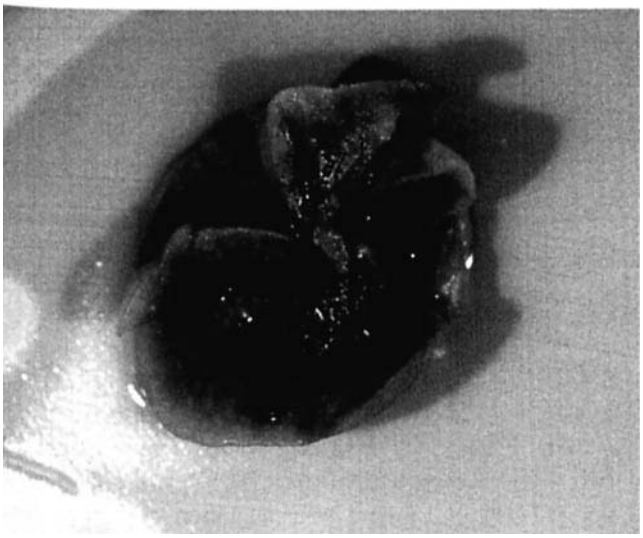

Figure 1. Lungs of 298-day-old mouse showing foci of galactosidase-positive cells that have stained blue, demonstrating survival of donor cells in host mouse. Similar foci were present on the surface of the liver and brains of several mice sacrificed at various time points as shown in Table 1.

studies were approved by the Institutional Animal Care and Use Committee.

Groups of recipient mice were euthanized by carbon dioxide narcosis at various ages from Day 1 through Day 328 following birth.

Fetal Liver Cell Preparation. Rosa26 embryos at embryonic age 12.5 days postconception (E12.5 dpc) were used as liver cell donors. Pregnant Rosa26 donors were euthanized by cervical dislocation. Following disinfection of the abdomen with 70% ethanol, the peritoneal cavity was exposed with sterile instruments and the uterus excised and opened to expose the embryos within their yolk sac. The embryos were placed in a drop of sterile saline and the yolk sac and umbilicus were removed to allow exsanguination. Each embryo was then placed into a fresh drop of sterile saline and the embryonic abdominal cavity was opened. The livers were removed and extraneous, nonhepatic tissue was dissected away. The livers were rinsed several times in sterile saline followed by trypsinization for 15 min at 37°C in 5 ml of 0.05% solution.

The hepatocytes were resuspended by gentle pipetting and the trypsin quenched upon the addition of 10 m*M* cold Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Herndon, VA). The cell suspension was then filtered through a 100-μm filter and the filter rinsed with an additional 25 ml of media. After centrifugation at 750 *g* for 10 min at 4°C, the pelleted cells were resuspended in 15 ml of DMEM/F12 + 10% FBS, followed by a second filtration through a 40-μm filter and centrifugation at 750 *g* for 5 min at 4°C. The cells were rinsed in 15 ml of cold phosphate-buffered saline (PBS), pelleted, resuspended in 1 ml of PBS, and the cell concentration determined using a hemocytometer. The cell concentration was adjusted to 10^5 cells per μl. Hepatocytes from wild-type mice were similarly prepared and administered to fetuses of three dams as described below to serve as controls.

Embryonic Hepatocyte Injection. Pregnant recipient mice were anesthetized using a Vetamac isoflurane vaporizer (Vetamac, Inc., Rossville, IN), which delivered approximately 2% isoflurane in oxygen at a rate of 1 liter/min. The animals were placed in dorsal recumbency and gently secured to a heating pad. The abdominal regions were shaved and disinfected with 70% ethanol, followed by betadine solution. A midline incision was made through the skin, the peritoneal wall and the uterine horns, containing the E12.5 embryos, and gently externalized using cotton-tip applicators soaked in sterile saline. The uteri were kept constantly moist during the remainder of the procedure. Each embryo, visualized through the uterine wall, was punctured into the peritoneal cavity through the uterus with a 50-μm-diameter beveled glass pipette. A minimum of 1.5×10^5 fetal liver cells (FLCs) in a 1.5-μl volume was injected between the hind leg and the liver. Once all the embryos were injected, the uterus was reinserted into the peritoneal cavity with the cotton tip applicators. The peritoneal wall was sutured with 5–0 vicryl and 1 ml of preheated saline was injected into the peritoneal cavity of the dam. A 5–0 vicryl suture was then used to close the abdominal skin incision. The incision was swabbed with betadine solution and the animal given oxygen (2 liters/min) to enhance recovery. The pregnant dams recovered from the surgery and delivered litters at the expected times.

Whole-Mount Staining for the Detection of β-Galactosidase. Organs of 129/C57J recipient mice were fixed in 4% paraformaldehyde in PBS for 2 hrs, followed by three rinses with PBS and three 30-min rinses in X-gal buffer (2 m*M* $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 in PBS). Organs were then incubated in X-gal stain (5 m*M* potassium ferrocyanide, 1 mg/ml X-gal, 2 m*M* $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 in PBS) for up to 48 hrs at 37°C. After the development of stain, organs were rinsed three times for 30 min with PBS and stored in 70% ethanol. Organs were examined whole for evidence of staining. Histological sections of tissues were prepared from organs stained as whole mount for β-galactosidase and examined for staining.

Results

Following surgery, all dams recovered normally and completed gestation. At necropsy, visible blue foci indicating tissue stained for β-galactosidase (Fig. 1) were observed in the lungs, livers, and brains of some mice (Table 1). Staining of all three tissues was noted as early as 20 days. Microscopic examination of tissues showed a wider spectrum of tissues that stained positive for β-galactosidase (Table 2). All tissue types showed microscopic staining for β-galactosidase in all time groups, although groups beyond

Table 1. Presence of Grossly Visible Staining of Organs[a]

Days postbirth	N	Lung[b]	Liver[b]	Brains[b]
0–100	20	2	4	2
101–200	9	3	6	2
201–300	28	11	19	3
301–328	13	3	8	2

[a] Positive organs had grossly visible foci of stain for galactosidase. Other tissues, including thymus, heart, kidney, spleen, intestine, and ovary/testis were negative for such foci in all animals.
[b] Number of positive samples.

100 days had generally higher percentages of positively stained samples for all tissues.

In the spleen, cells stained for β-galactosidase were primarily hematopoietic cells. This population of cells included cells from both the leukocytic and erythrocytic lineages. Similarly, the thymuses of several mice contained positively stained lymphocytes. In contrast to the spleen and thymus, positively stained cells in other tissues, including liver, brain, and lung, existed as islands of polyhedral cells having round nuclei with prominent nucleoli, morphologically similar to hepatocytes (Figs. 2 and 3). In some cases, the cells were forming small cords, typical of hepatocytes. Inflammation was not associated with these cells in any tissue examined.

Several areas of bronchial epithelium were noted to stain for β-galactosidase. It is important to note that positively stained cells in the gonads were exclusively represented by cells in the interstitium and not within the spermatogenic lineage. These cells had the morphologic appearance of normal interstitial connective tissue cells and did not appear to be hepatocytes.

The intestine and kidney of control mice had significant numbers of positively stained cells; thus, it was difficult to determine the extent to which staining of intestine and kidney tissue from test mice represented background staining.

Discussion

Transplantation of pluripotent cells *in utero* has been suggested as a clinical approach for treatment of congenital disorders that result from deficiency of specific cells and cell-produced factors (16). Indeed, feasibility of this approach has been demonstrated in humans by transplantation of fetal liver stem cells *in utero* in an attempt to correct severe immunodeficiency disease and thalassemia major (17–19). Successful transplantation of human fetal liver cells into sheep, baboons, and mice suggests that xenogeneic transplantation *in utero* could eventually find clinical application (20–22). For example, allogeneic normal fetal liver cells administered into the blood vessels of the fetal placenta corrected inherited macrocytic anemia in mice with recipient mice becoming tolerant of donor cells (23). Taylor *et al.* (24) found that allogeneic fetal liver cells had a selective advantage over adult bone marrow cells when administered to fetal severe combined immunodeficient mice recipients.

Engraftment of fetal hematopoietic cells given by intraperitoneal (ip) injection *in utero* was demonstrated by karyotype analysis of peripheral blood leukocytes and bone marrow in rhesus monkeys (25). Although it has been demonstrated that liver-derived fetal stem cells preferentially home to the recipient liver and spleen early in development and to the bone marrow later in ontogeny (22, 26), the potential of transplanted cells to home to other, nonhematopoietic sites has not been investigated. The idea that grafted cells may home to other sites could have significant importance for the utility of fetal liver cell transplants if the cells have the potential to home to sites where they would not be normally found.

Following ip administration of syngeneic fetal liver cells at Day 12.5 of gestation, we found that individual and foci of donor cells were subsequently observed in a variety

Table 2. Presence of Microscopic Foci Stained Positive[a]

Days postbirth	N	Thymus	Lung	Liver	Kidney	Spleen	Intestine	Brain	Gonad
0–100	20	10(50)	2(10)	5(25)	13(65)	9(45)	15(75)	2(10)	12(60)
101–200	9	9(100)	3(33)	7(78)	9(100)	9(100)	9(100)	2(22)	9(100)
201–300	28	27(96)	19(68)	22(79)	27(96)	26(93)	27(96)	6(21)	28(100)
301–328	13	13(100)	3(23)	8(62)	13(100)	13(100)	13(100)	2(15)	13(100)

[a] Results indicate numbers of samples found to have positive staining microscopically for β-galactosidase. The percent of positive samples out of the total samples per time point is in parentheses. Data were analyzed by chi-square analysis. Significantly fewer samples stained positive for β-galactosidase at 0–100 days compared with older age groups for thymus, liver ($P \leq 0.001$), kidney ($P \leq 0.01$), spleen ($P \leq 0.01$), intestine ($P \leq 0.025$), and gonad ($P \leq 0.001$) samples; significantly more ($P \leq 0.001$) samples were positive for β-galactosidase in the lung at 201–300 days compared with all other age groups, and there were no significant differences between age groups in presence of β-galactosidase positive brain samples.

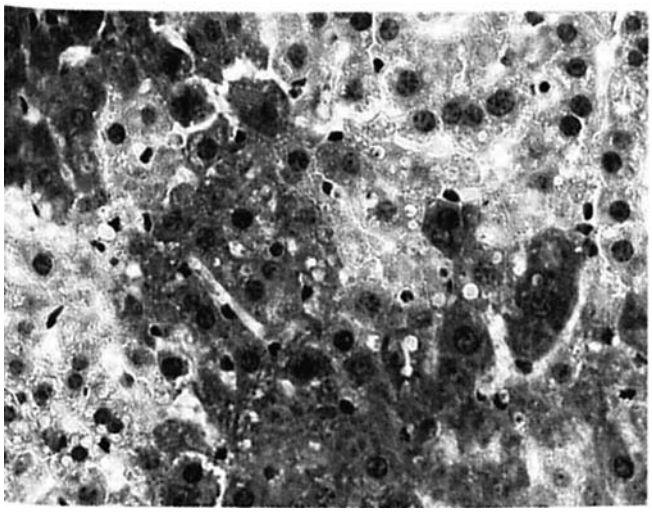

Figure 2. Focus of galactosidase-positive (blue-stained) cells in the liver. The cells are morphologically similar to adjacent hepatocytes, with a polyhedral shape, round nuclei and prominent nucleoli. Magnification ×400.

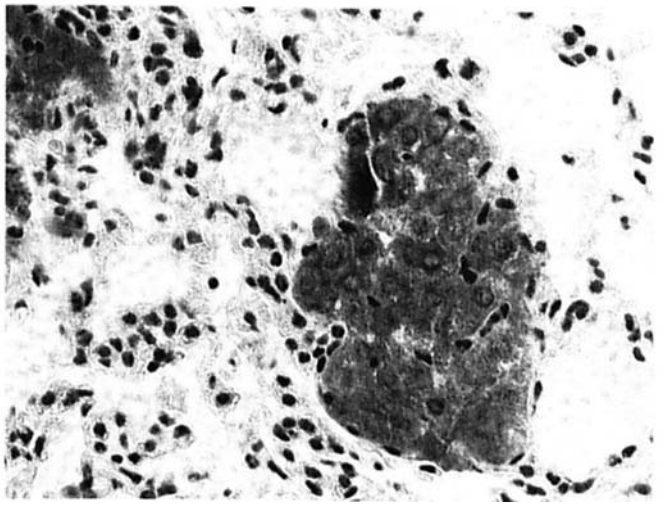

Figure 3. Focus of galactosidase-positive (blue-stained) cells in the lung. The galactosidase-positive cells have a morphologic appearance similar to the hepatocytes shown in Figure 2. Magnification ×400.

of tissues. As early as 20 days of age, recipient-derived cells were found in the thymus, lung, liver, spleen, brain, and gonad. Although positive cells were found earlier in the intestine and kidney, this may represent staining for native β-galactosidase in those tissues (27, 28). In the oldest age group (201–300 days), decreased staining was observed in lung, liver, and brain. A lack of data exists with respect to changes in β-galactosidase activity with age, but it has been shown that β-galactosidase activity of the submandibular gland decreases with age in rats (29). Conversely, proliferatively senescent cultured fibroblasts had reduced β-galactosidase activity (30). Although additional work is required to define the precise mechanism, it may be that the reduced β-galactosidase activity we observed in some tissues is related to biochemical maturation of the tissue.

Engraftment and site-specific differentiation of human mesenchymal stem cells into chondrocytes, adipocytes, myocytes, and thymic stroma has been demonstrated following *in utero* administration in sheep (31). We found foci of cells resembling hepatocytes in lung and brain, suggesting that nonhematopoietic cells colonized within these tissues. Although affected mice were clinically normal, the possibility exists that foci of transplanted cells could adversely affect the function of host tissue. This outcome might be lessened in larger species, however, in which it is logistically more feasible to precisely deliver transplanted cells to fetuses via the portal vein *in utero*. Additionally, the simple presence of transplanted cells located in tissues other than their normal *de novo* location would not necessarily result in diminished function of those tissues. Our observation of β-galactosidase-positive cells from the hematopoietic lineage is consistent with the role of the fetal mouse liver as a hematopoietic organ. Hematopoiesis in mouse fetal liver begins at 10 days of gestation and begins to decline after 15 days of gestation (32). Because the hepatocyte donor fetuses were taken at 12.5 days of gestation, it is apparent that hematopoietic cells were transferred along with hepatocytes to recipients.

Allogeneic hepatocyte transplantation in mice is limited by immunological responses to the transplanted cells even when transplantation occurs *in utero* (33, 34). When administered via the portal circulation, allogeneic cells stimulate weak humoral and strong cell-mediated immune responses (35). In contrast, our results demonstrate that syngeneic transplants are tolerated when performed *in utero*. The lack of an inflammatory response to the islands of β-galactosidase-positive cells supports this conclusion. Because both donor and recipient mice were of the same strain background, this result is not unexpected.

In summary, we found that *in utero* administration of syngeneic fetal liver cells to mice resulted in tropism of donor cells for a variety of tissues in the host. These cells were identified as early as 20 days after birth and persisted through at least 328 days of age. The possibility that cells administered *in utero* may inadvertently colonize tissues other than those intended as targets suggests that application of *in utero* cell transplantation should be approached with caution regarding possible interference with the function of nontarget tissues.

We thank Mayra Sandoval-Cooper and Juan Feng for technical assistance.

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