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# Long-term effects of intrasplenically transplanted adult hepatocytes and fetal liver in hyperbilirubinemic Gunn rats

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**Abstract** We performed adult hepatocyte transplantation (HCTx) and fetal liver transplantation (FLTx) into the spleens of hyperbilirubinemic Gunn rats in congenic combination and we compared the long-term effects of these procedures for as long as 12 months. Proliferative activity of intrasplenic hepatocytes was evaluated using anti-proliferating cell nuclear antigen (PCNA) immunohistochemical staining. The serum total bilirubin levels (T. Bil) significantly decreased from 7.16 ± 0.25 mg/dl to 4.38 ± 0.60 mg/dl 2 months after HCTx and gradually decreased thereafter until 12 months after transplantation (3.23 ± 0.37 mg/dl, $P < 0.05$ vs preoperative value). The T. Bil change after FLTx was similar to that of HCTx: 7.22 ± 0.24 mg/dl before FLTx, and 4.92 ± 0.24 and 3.06 ± 0.47 mg/dl, 2 and 12 months after FLTx ($P < 0.05$), respectively. Bilirubin glucuronides, which were not detectable in the bile from untreated Gunn rats, appeared in considerable amounts 4 months after HCTx and FLTx (27.5 % and 36.0 % of total bile, respectively). PCNA labeling indices of intrasplenic hepatocytes (4.9 % ± 0.9 % and 3.7 % ± 0.7 %, 6 months after HCTx and FLTx, respectively) were slightly higher than those of normal hepatocytes (1.0 % ± 0.1 %) in the host liver. In conclusion, both adult and fetal rat hepatocytes transplanted into the spleen in congenic combination functioned for at least a year in terms of bilirubin glucuronidation. The spleen is considered to be one of the optimal grafting sites for hepatocytes, with nearly lifelong significant function and proliferative activity.

**Key words** Gunn rat, hepatocyte transplantation · Hepatocyte transplantation, Gunn rat · Intrasplenic transplantation, hepatocytes

## Introduction

Gunn rats have a congenital deficiency of bilirubin-uridine diphosphate glucuronyltransferase activity and are unable to glucuronidate bilirubin in the bile, resulting in lifelong, nonhemolytic, unconjugated hyperbilirubinemia [11]. Monitoring serum total bilirubin levels as an indicator of hepatic graft function, this model has widely been used in experimental liver transplantation [1] and adult hepatocyte transplantation to various sites including the peritoneal cavity [5, 6], spleen [25], portal vein [10, 12], and subcutaneous tissue [23]. Most of these studies were performed in allogenic combination, which requires immunosuppression, and graft function was followed up for relatively short periods (up to 12 weeks). In 1986, congenic enzyme-deficient and non-enzyme-deficient rats were established and graft function was followed up for 30 weeks after intrasplenic hepatocyte transplantation without immunosuppression [26].

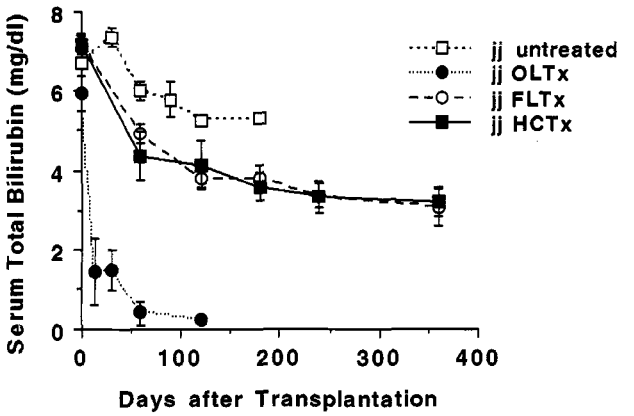


**Fig. 1** Change in serum total bilirubin levels (*T. Bil*) in Gunn rats after hepatocyte (*HCTx*), fetal liver (*FLTx*), and orthotopic liver (*OLTx*) transplantation

Fetal liver cells are highly regenerative and are known to grow and function for a long period after intrasplenic transplantation [20]. Aside from our preliminary report [18], there have been few reports on the effect of fetal liver transplantation in Gunn rats [2].

In this study, we performed adult hepatocyte transplantation (HCTx) and fetal liver transplantation (FLTx) into the spleens of Gunn rats in congenic combination and compared the effects of these procedures. Since it is essential to show long-term preservation of hepatocyte specific functioning of the graft before considering clinical application of these transplantation techniques [9], we extended the follow-up period to 12 months. Untreated Gunn rats, which underwent orthotopic liver transplantation (OLTx), and normal congenic rats served as controls. The proliferative activity of intrasplenic hepatocytes was also evaluated via a proliferating cell nuclear antigen (PCNA) labeling index. To our knowledge, this is the longest and the most thorough investigation on the effects of hepatocyte and fetal liver transplantation in Gunn rats.

## Materials and methods

### Animals

Hyperbilirubinemic Gunn rats [11] (jj, $RT1^k$), weighing 130–170 g, were used as recipients, and normal congenic rats (Wistar Shi) as donors. Having an identical genetic make-up, except for the bilirubin-uridine diphosphate (UDP)-glucuronyl-transferase locus, these two strains were bred and maintained at Shionogi Abrahi Laboratory (Shiga, Japan). All animals were acclimatized to a 12-h light and dark cycle for at least 1 week prior to the experiment. Water and standard laboratory chow were administered ad libitum.

### Techniques of transplantation

Adult hepatocytes were isolated using a two-step collagenase perfusion technique described previously [13, 22]. The cell suspension was subjected to low-speed centrifugation three times to yield a final fraction enriched with viable hepatocytes ( > 85 %, trypan blue exclusion). Approximately $10^7$ hepatocytes in 0.2 ml Hank's solution were injected into the recipient's spleen using 25-gauge needles. The hilar vessels of the spleen were clamped during injection. Fetal livers were taken at 20 days of gestation, when the volumetric fraction of hepatocytes is known to go up to 66 % [24]. Approximately 0.2 ml of minced liver tissue suspension in Hank's solution was injected into the spleen with 22-gauge needles. Orthotopic liver transplantation was performed according to the method of Lee et al. [15]. Cuffs were used for anastomosing the portal vein, infrahepatic vena cava, and common bile duct. All operations were done under ether anesthesia.

### Assessment of hepatocyte function

Blood samples were taken from normal congenic, untreated, and transplanted rats at certain intervals for up to 12 months. Serum total bilirubin (T. Bil) was measured with an auto-analyzer (Hitachi model 726). Bile samples were obtained at 4 months after transplantation. The common bile duct was cannulated with fine polyethylene tubing (PE-10, Clay Adams, Parsippany, N.J., USA) and a bile sample was collected for 40 min in dim light, mixed with 20 % ascorbic acid (4:1 v/v), and stored at –80 °C. Bilirubin fractions of the bile were analyzed using HPLC [29].

### Histological and morphometric analysis

Splenectomy was performed at certain intervals and the specimens were processed for hematoxylin and eosin (H&E) or periodic acid-schiff (PAS) staining. Anti-PCNA immunohistochemical staining was also applied to evaluate the proliferative potential of transplanted hepatocytes. After deparaffinization and rehydration, 4-μm sections of the spleen were incubated in 3 % hydrogen peroxidase for 10 min. Normal goat serum was applied for blocking for 10 min. The slides were incubated with anti-PCNA antibody (PC-10, DAKO A/S, Glostrup, Denmark) for 10 min and then incubated for an additional 10 min with a biotinylated goat anti-mouse IgG. This was followed by incubation with avidin DH-biotinylated horseradish peroxidase H complex (Nichirei, Tokyo, Japan). Finally, sections were developed with 0.02 % diaminobenzine tetrahydrochloride. All incubations were done at room temperature. The labeling index was calculated after examining 100–200 intrasplenic hepatocytes that were morphologically distinguishable from spleen cells.

To estimate cerebellar atrophy, which is one of the pathological features of Gunn rats, the cerebellum was fixed with 10 % formalin perfusion through the heart and was weighed 4 months after transplantation.

### Statistical analysis

All values shown represent the mean ± SEM. Student's *t*-test was used for comparing group means. The level of significance was established as a $P$ level below $< 0.05$.

## Results

### Change in the serum total bilirubin (Fig. 1)

Gunn rats tolerate hepatocyte and fetal liver transplantation well; survival rates at 6 and 12 months were 78.9 %

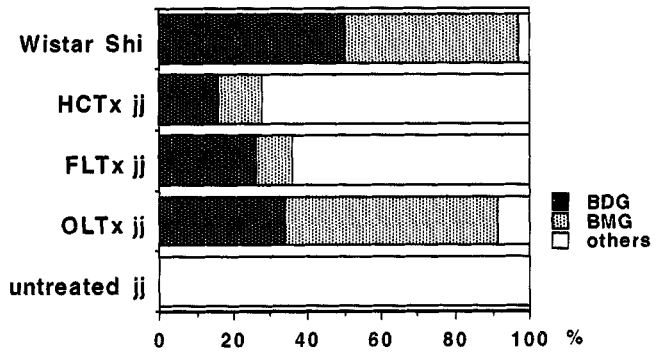


**Fig. 2** Bilirubin fractions in the bile from Gunn rats 4 months after hepatocyte (*HCTx*), fetal liver (*FLTx*), and orthotopic liver (*OLTx*) transplantation. Each *bar* represents the mean from four to six animals (*BDG* bilirubin diglucuronide, *BMG* birilubin monoglucuronide, *Others* unconjugated bilirubin and unknown peaks)

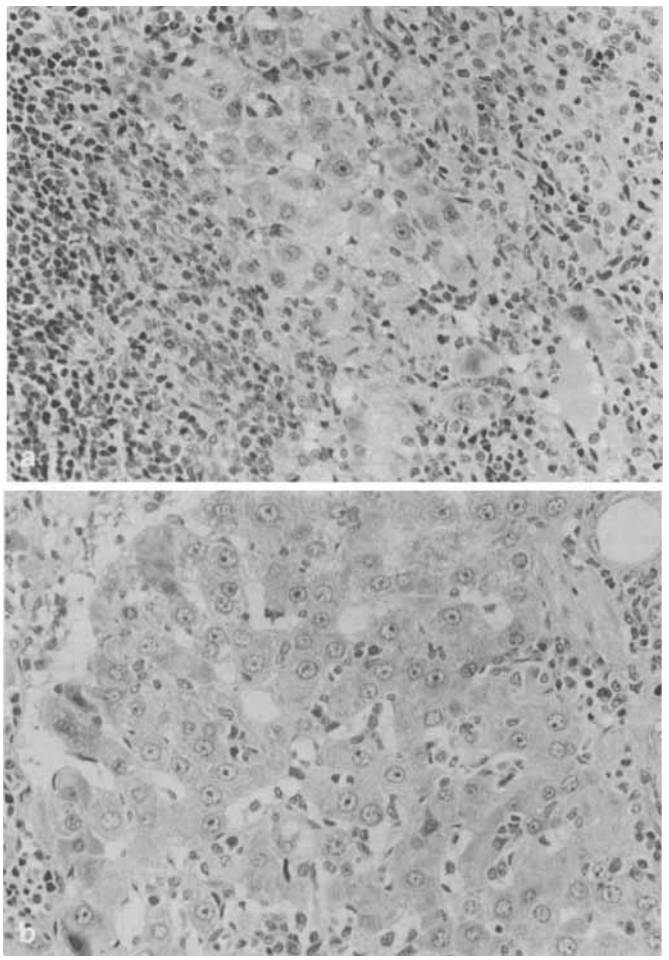

**Fig. 3a, b** Photomicrograph of intrasplenic hepatocytes **a** 1 month and **b** 6 months after hepatocyte transplantation (H&E × 110)

and 73.8 % after HCTx, and 87.5 % and 68.9 % after FLTx. The serum T. Bil before HCTx was 7.16 ± 0.25 mg/dl and it significantly decreased to 4.38 ± 0.60 mg/dl 2 months after HCTx ($P < 0.05$). It gradually decreased thereafter, and T. Bil levels 6 and 12 months after HCTx were 3.59 ± 0.35 and 3.23 ± 0.37 mg/dl ($n = 12$). The serum T. Bil change after FLTx was similar to that after HCTx: 7.22 ± 0.24 mg/dl before FLTx, and 4.92 ± 0.24 and 3.06 ± 0.47 mg/dl 2 months and 12 months after FLTx, respectively ($n = 12$, $P < 0.05$ vs preoperative value). The improvement in hyperbilirubinemia was not statistically different between HCTx and FLTx.

The serum T. Bil level was almost completely normalized after OLTx: 0.40 ± 0.30 mg/dl 2 months after transplantation ($n = 6$, $P < 0.05$ vs preoperative value). The T. Bil level of untreated normal Wistar Shi rats was 0.27 ± 0.01 mg/dl ($n = 5$) and it did not change significantly during 6 months of follow-up.

## Bilirubin fractions in the bile (Fig. 2)

Bilirubin fraction analysis of the bile from untreated Gunn rats showed a completely different pattern from that of the bile from normal Wistar Shi rats. Bilirubin glucuronides (BMG, BDG) were essentially undetectable in the bile from untreated Gunn rats, whereas they constituted 96.8 % of the total bile from normal Wistar Shi rats. Bilirubin glucuronides appeared in considerable amounts in the bile from Gunn rats 4 months after HCTx (27.5 % of total) and FLTx (36.0 %). The bilirubin fraction pattern of Gunn rats 4 months after OLTx was almost normal.

## Morphology of intrasplenic hepatocytes

Hepatocytes transplanted into the spleen scattered in the splenic red pulp until 4 weeks after transplantation. PAS-positive hepatocyte colonies appeared either microscopically or macroscopically 4–8 weeks after transplantation (Fig. 3 a). These colonies gradually grew thereafter, forming a cord-like structure (Fig. 3 b).

The fetal liver is a mixture of immature hepatocytes with a bright cytoplasm and hematopoietic cells. Fetal liver cells decrease after intrasplenic transplantation, and mature hepatocyte colonies were detectable 4 weeks after transplantation.

## PCNA labeling index of intrasplenic hepatocytes (Table 1)

The PCNA labeling index of normal quiescent hepatocytes in the host liver was 1.0 % ± 0.1 %. The labeling index of intrasplenic hepatocytes was very low until

**Table 1** PCNA labeling index (%) of intrasplenic hepatocytes

| | Before transplantation[a] | 1 week | 2 weeks | 4 weeks | 2 months | 6 months |
|---|---|---|---|---|---|---|
| HCTx | 1.0 ± 0.1 | 4.5 ± 5.2 | N.D.[b] | 1.5 ± 1.0 | 4.4 ± 0.6 | 4.9 ± 0.9 |
| FLTx | 42.0 ± 6.4 | 45.0 ± 4.4 | 20.9 ± 4.0 | 15.7 ± 8.6 | 7.8 ± 1.0 | 3.7 ± 0.7 |

[a] PCNA labeling index of normal adult or fetal hepatocytes
[b] Not determined due to very small number of intrasplenic hepatocytes

**Table 2** Cerebellar weight 4 months after transplantation

| | Cerebellar weight (mg) |
|---|---|
| Wistar Shi ($n = 6$) | 289.9 ± 5.0 |
| HCTx (jj) ($n = 4$) | 101.0 ± 6.0* |
| FLTx (jj) ($n = 6$) | 108.6 ± 2.3* |
| OLTx (jj) ($n = 5$) | 93.4 ± 6.8* |
| untreated Gunn rat (jj) ($n = 5$) | 98.9 ± 7.4* |

* $P < 0.05$ vs Wistar Shi

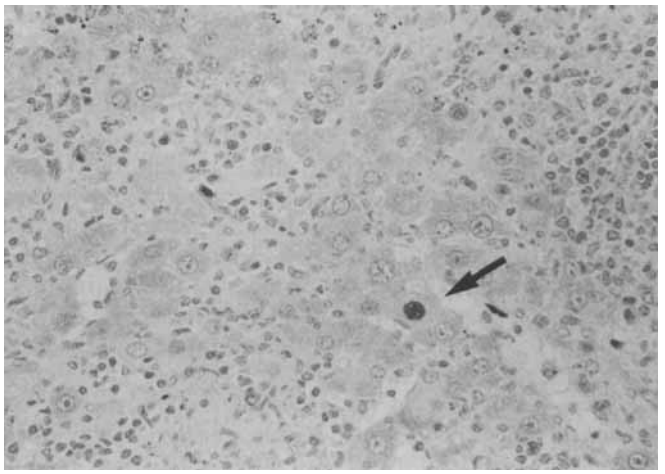

**Fig. 4** Anti-PCNA immunohistochemical staining of intrasplenic hepatocytes 6 months after hepatocyte transplantation (×110). Note the positively stained nucleus in a hepatocyte colony (*arrow*)

4 weeks after transplantation (1.5 % ± 1.0 %), and it gradually increased to 4.9 % ± 0.9 % 6 months after the treatment (Fig. 4).

The PCNA labeling index of fetal hepatocytes was as high as 42.0 % ± 6.4 %, and it gradually decreased after transplantation. The labeling index 2 and 6 months after fetal liver transplantation was 7.8 % ± 1.0 % and 3.7 % ± 0.7 %, respectively.

### Macroscopic findings of the cerebellum

Macroscopically, cerebellums from all experimental groups of Gunn rats, whether or not they had been treated with any technique, were clearly smaller than those from normal Wistar Shi rats. The cerebellar weight of untreated Gunn rats was 98.9 ± 7.4 mg, which was around one-third of the normal value. The cerebellar weight of HCTx-, FLTx-, and OLTx-treated Gunn rats was around the same level (Table 2). None of the transplantation techniques, including OLTx, succeeded in reducing the cerebellar atrophy of Gunn rats.

## Discussion

The Gunn rat is a mutant that lacks bilirubin-UDP-glucuronyl-transferase activity and is unable to glucuronidate bilirubin in the bile, resulting in unconjugated hyperbilirubinemia [11]. Since the study by Groth et al. [10], this model has widely been used in experimental liver and hepatocyte transplantation, monitoring serum total bilirubin levels as an indicator of graft function [3, 23, 25].

In this study, we have shown that the serum total bilirubin levels of Gunn rats were significantly decreased after both adult hepatocyte and fetal liver transplantation in similar manners. These improvements were noticed around 2 months after the treatments and they reached their peaks 6–12 months post-transplantation. Compared to the results of intraportal injection of hepatocytes [10], these effects appeared at a slightly slower rate but lasted much longer at their full strength. This metabolic improvement was also demonstrated by increased bilirubin glucuronide fractions in the bile.

We have previously reported that serum albumin synthesized by intrasplenic grafts in Nagase's analbuminemic rats corresponded to 1 %–2 % of the normal value [18, 20]. Hepatocyte graft mass as big as 1 %–2 % of the whole liver may, thus, have provided the partial improvement in hyperbilirubinemia in Gunn rats presented in this study. This estimation appears reasonable since as little as 12 % of the whole liver could provide almost complete normalization of hyperbilirubinemia in Gunn rats after partial liver transplantation [1].

Recently, there have been a few efforts to increase the number of transplanted hepatocytes using a selective intraportal injection technique [12] or by setting up a subcutaneal solid support system [23]. However, these methods do not resolve the problem of portal embolism or massive early loss of the implanted hepato-

cytes. Some technical breakthrough to increase graft volume may be needed for the future clinical application of hepatocyte transplantation as a hepatic support.

The morphology of intrasplenic hepatocytes observed microscopically was consistent with the reports by Mito et al. [7]. Hepatocytes in the spleen rapidly decreased in the first 2 weeks post-transplantation, reappeared as cell colonies 4–8 weeks after treatment, and gradually increased in number thereafter, forming a trabecular pattern. Early loss of the hepatocyte graft may be due to postimplantation death of a certain population of hepatocytes.

The proliferative activity of intrasplenic adult hepatocytes has been evaluated by [$H^3$]-thymidine labeling [17] and bromodeoxyuridine (BrdU) labeling indices [3]. Monoclonal antibodies have recently been developed against PCNA, an auxiliary factor for the DNA polymerase ∂ [8]. The immunohistochemical detection of PCNA has been found to depend on the proliferative potential of a number of cell types including hepatocytes [28]. A good correlation has been reported between nuclear labeling for BrdU and PCNA in rat hepatocytes [28]. We therefore applied this method to estimate hepatocyte proliferation in the spleen [14]. Slightly higher PCNA labeling indices in transplanted hepatocytes and fetal livers than in host livers after transplantation indicate a slow but steady growth of the intrasplenic graft after transplantation. Possible growth stimulators in the spleen in this setting include transforming growth factor α, produced by the hepatocyte itself [16], hepatocyte growth factor produced by platelets or intrasplenic leukocytes [19], and extracellular matrices inside the spleen [27]. These remain to be clarified. It is not known why, in the presence of continuing proliferation of intrasplenic hepatocytes 6 months after transplantation, the glucuronidating function reaches a plateau. This may be due to an increased renewal rate or a shortened life of intrasplenic hepatocytes, or due to decreased enzyme production per cell. Further study may elucidate the mechanisms of this phenomenon. In accordance with a report on the PCNA labeling index of neonatal rat livers [4], the PCNA labeling index of fetal livers was as high as 42.0 % ± 6.4 % before transplantation. The gradual decrease in the PCNA labeling index of fetal hepatocytes after transplantation may be due to cell maturation or loss of local growth stimulants.

Cerebellar hypoplasia is one of the most prominent morphological features of Gunn rats [21]. Although both HCTx and FLTx performed at the age of 2 months or older partially corrected hyperbilirubinemia, neither corrected the disorders in the cerebellum. OLTx, which completely corrected hyperbilirubinemia in Gunn rats, did not have any effect on the cerebellum either. For the prophylaxis of cerebellar hypoplasia, treatments should be commenced earlier, probably in the neonatal period, when cerebellar hypoplasia becomes prominent [21].

In conclusion, both adult hepatocyte and fetal liver transplantation into the spleen were effective in correcting a metabolic abnormality in Gunn rats in similar manners for as long as 1 year. This longevity of grafted hepatocytes with significant specific function and some proliferative activity was accomplished by choosing the spleen as the grafting site. Since these corrections were only partial compared to those achieved by orthotopic liver transplantation, some measures to increase graft mass may be needed for the future clinical application of hepatocyte transplantation.

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