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Liver Regeneration (2012.05) :123~140.

Analbuminemic Rat Model for Hepatocyte Transplantation

Ogawa Katsuhiro、Inagaki Mitsuhiro

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Katsuhiro Ogawa and Mitsuhiro Inagaki

*Departments of Pathology & Surgery, Asahikawa Medical University,  
Japan*

## 1. Introduction

Orthotopic whole and split liver transplantation are successful and well-established treatments for liver disease. However, the short supply of donor organs is a major obstacle to the widespread use of this therapy (Merion, 2010). Recent research has focused on cell transplantation as a therapy for liver disease. Although only a small number of hepatocytes can be transplanted into the liver by transfusion into the portal circulation, the transplanted cells can regenerate the normal hepatic tissue when liver growth is impaired as a result of continuous hepatic damage (Roy-Chowdhury & Roy-Chowdhury, 2011; Gilgenkranz, 2010). A number of animal models have demonstrated that much of the liver can be replaced via repopulation by a small number of transplanted hepatocytes, which restore normal liver functions and improve recipient survival. Hepatocyte transplantation is therefore expected to be used as a therapy for human liver disease. To optimize the therapeutic application of hepatocyte transplantation, however, number of problems such as the methods to monitor and evaluate the functionality of the transplanted cells and the suppression of host immunological responses against the transplanted cells require mitigation.

The cell transplantation model, which uses Fischer344 (F344) rats as donors and F344 congenic analbuminemic (F344-alb) rats as recipients, provides an excellent system for the investigation of cell transplantation, because no immunosuppressants are required for cell transplantation. In addition, the transplantability can be accurately evaluated with immunohistochemistry to stain for albumin, using PCR to detect albumin mRNA and genomic DNA, and by measuring serum albumin protein. In this review, we describe the hepatocyte transplantation system that uses F344 and F344-alb rats to study the intrahepatic hepatocyte transplantation.

## 2. Nagase analbuminemic rats (NARs)

Analbuminemic rats (Nagase analbuminemic rats, NARs) were first established from Sprague Dawley (SD) rats by Nagase et al. (1979). NARs show extraordinarily low serum albumin, hyperlipidemia and hormonal changes. However, their growth and reproduction rates do not differ from those of normal rats. The amount of total serum protein in NARs is similar to that seen in normal rats due to the increase in proteins other than albumin.

Analbuminemia in NARs is an autosomal recessive trait. Therefore, the serum albumin levels are nearly normal in the F1 hybrids of NARs and normal rats.

The molecular basis for analbuminemia in NARs is a deletion of base 5 to base 11 at the 5' end of the 9th intron of the albumin gene (Figure 1A a) (Esumi et al., 1983). Most of the albumin mRNA in NARs shows a precise deletion of exon H, which is skipped during albumin pre-mRNA processing following the deletion in the 9th intron (Figure 1A b) (Shalaby & Shafritz, 1990). The deletion of exon H causes a frameshift in the mRNA and the occurrence of a translation termination signal at the 7th codon of exon I, preventing production of the albumin protein (Shalaby & Shafritz, 1990).

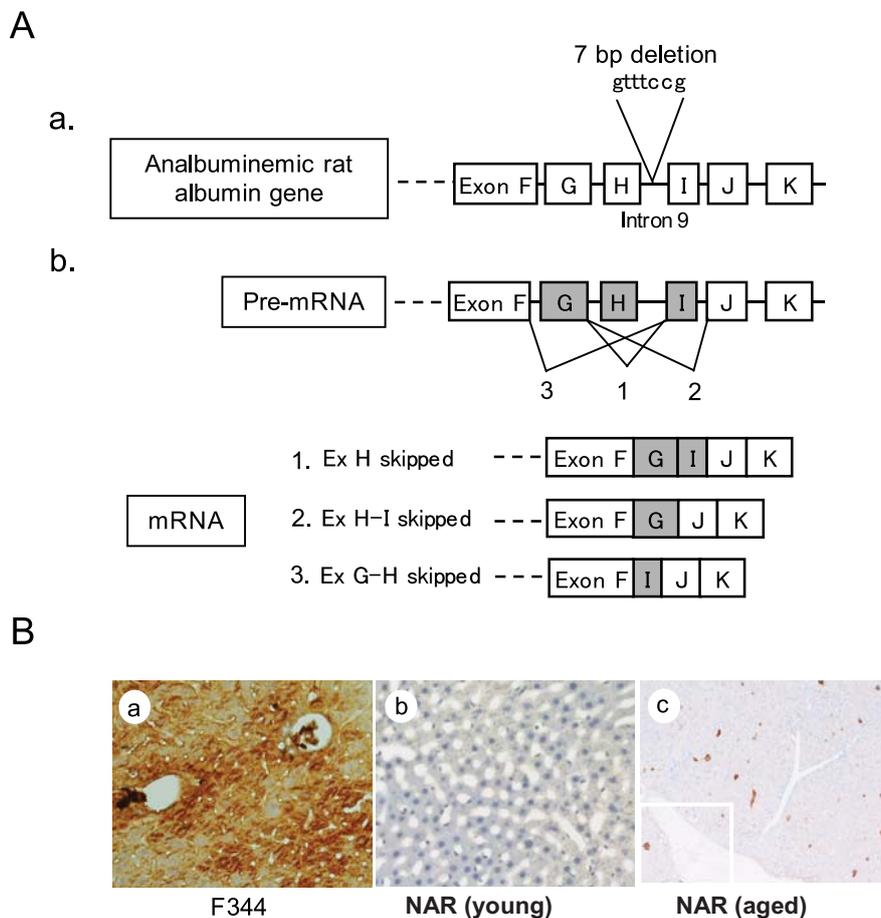


Fig. 1. A. The structures of the albumin gene and mRNA in the Nagase analbuminemic rat (NAR). a. Seven base pairs (the 5th to 11th base pairs from the start of the intron) are deleted between exons H and I (9th intron). b. Exon H is skipped in most albumin mRNAs during albumin pre-mRNA processing (1). With aging and treatment with hepatocarcinogens, the levels of albumin mRNA transcripts that skip exons H and I (2) and exons G and H (3)

increase. B. Immunostaining for albumin in an F344 rat liver (a) and in young (b) and aged NAR livers (c). Albumin-positive hepatocytes are visible in the aged NAR liver (c) and are usually present as single cells (inset) or two-cell clusters.

In contrast to the hepatocytes of normal rats (Figure 1B a), hepatocytes of NARs are generally negative for immunohistochemical staining of albumin (Figure 1B b). However, although NARs display extremely low serum albumin, albumin-positive hepatocytes sometimes can be seen in the liver tissue of NARs at low frequency. The number of such albumin-positive hepatocytes increases with aging (Figure 1B c) and after treatment with hepatic carcinogens (e.g. 3'-methyl-4-dimethylaminoazobenzene) (Makino et al., 1986). These albumin-positive hepatocytes are present as single or double cells in cross sections (Figure 1B c inset) and rarely form clusters consisting of more than three cells. These cells remain as single or double cells after liver regeneration following two thirds hepatectomy (PH), suggesting that albumin-positive hepatocytes may have a low proliferative capacity. Under these conditions, the prevalence of albumin mRNAs missing exons G and H and exons H and I increase along with exon H-skipped albumin mRNA (Figure 1A b). In addition, aberrant 60-kD albumin is generated in the liver (Kaneko et al., 1991). This abnormal albumin may be a translation product of mRNA that skips exons H and I, and may accumulate in the cytoplasm because of defects in extracellular albumin secretion, resulting in positive albumin immunostaining (Kaneko et al., 1991).

### 3. F344-congenic analbuminemic rats (F344-alb rats)

Although NARs are derived from SD rats, the SD rats consist of out-bred strains. Therefore, SD rats may be genetically heterogeneous. We first tried to transplant SD rat hepatocytes into an NAR liver without immunosuppressants. However, this experiment was unsuccessful, most likely because the SD rats were immunogenetically heterogeneous. Co-cultured spleen cells from NARs and SD rats displayed a higher rate of cell proliferation compared to cells in NAR/NAR or SD/SD cultures (Yokota & Ogawa, 1978). These results indicate that the immunological rejection may occur after the transplantation of the SD hepatocytes into the NAR liver.

In addition to the variety of abnormalities in serum proteins, lipids and hormones, NARs differ from SD rats in their susceptibility to tumorigenesis induced by chemical carcinogens in various organs. Notably, NARs are highly susceptible to urinary bladder tumors that are induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) (Kakizoe et al., 1982) and less susceptible to hepatocarcinogenesis induced by diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) (Asamoto et al., 1989). It is not clear whether the difference in the susceptibility to chemical carcinogens is the result of the analbuminemia in NARs or of differences in the genetic backgrounds of NARs and SD rats. To reduce the genetic variability, Takahashi et al. (Takahashi et al., 1988) established F344-alb rats with the genetic background of F344 rats. F344-alb rats are highly susceptible to BBN-induced urinary tumors compared to F344 rats (Takahashi et al., 1988) and are equally sensitive to DEN-2-AAF-induced hepatocarcinogenesis (Ohta et al., 1994). Because the only genetic difference between F344-alb rats and F344 rats is the aforementioned 7-base-pair deletion in the albumin gene in the F344-alb rats, the pairing of F344 and F344-alb rats can be used for cell transplantation without using immunosuppressants.

#### 4. Hepatocyte transplantation using F344 and F344-alb rats

To monitor the transplantability of hepatocytes in the liver, transplanted cells are detected using markers that are specifically expressed in the donor cells. For this purpose, various immunohistochemical and enzymatic histochemical procedures are used to visualize the proteins that are specifically expressed in the donor hepatocytes, such as *E. coli*  $\beta$ -galactosidase ( $\beta$ -gal) (ROSA26-transgenic mice) (Mao et al., 1999) or green fluorescence protein (GFP-transgenic mice) in the normal mouse liver (Chiocchetti et al., 1997), dipeptidyl peptidase IV (DPPIV) in the DPPIV<sup>-/-</sup> rat liver (Gupta et al., 1995) and fumarylacetoacetate hydrolase (FAH) protein in the FAH<sup>-/-</sup> mouse liver (Hamman et al., 2005). When combining cells from a male donor and a female recipient, Y chromosome-specific *in situ* hybridization techniques are also useful (Eckert et al., 1995).

In the F344/F344-alb model, albumin-positive donor F344 hepatocytes in the F344-alb liver are detected using immunohistochemical staining of albumin and PCR-based techniques detecting normal albumin mRNA and genomic DNA. In addition, the functionality of F344 hepatocytes can be repeatedly evaluated by taking blood samples and examining the elevation of serum albumin levels.

In immunohistochemical staining for albumin, the technique used for tissue fixation is important because albumin tends to diffuse out of hepatocytes during tissue fixation. We usually fix the liver tissues by perfusing periodate-lysine-paraformaldehyde (PLP) fixative through the portal vein. We then incubate the sliced hepatic tissues in the same fixative overnight at 4°C and then embed the tissues in paraffin (Ogawa et al., 1993). These procedures preserve the antigenicity of albumin and the morphological integrity of hepatic tissues. Antibodies that specifically recognize rat albumin are commercially available.

Donor F344 hepatocytes can also be detected by RT-PCR targeting normal albumin mRNA (Ohta et al., 1993a). Because albumin mRNA in F344-alb rats lacks exon H, RT-PCR with primers targeting exons G and I amplify both the normal and the short albumin mRNA from RNA that is isolated from recipient livers. In addition, primers targeting exons H and I specifically amplify normal albumin mRNA. Because the expression levels of exon H-skipped albumin mRNA in the F344-alb liver are low but relatively constant, the small amounts of normal albumin mRNA in the recipient F344-alb rat livers can be quantified using abnormal albumin mRNA as an internal standard (Ohta et al., 1993a).

The donor-derived F344 hepatocytes can also be detected by the amplification of the albumin genomic DNA sequences using DNA isolated from the recipient livers (Ogawa et al., 1993). Because of the 7-base-pair deletion in the 9th intron of the analbuminemic albumin gene, PCR amplification of amplicons spanning these sequences can differentially detect normal and abnormal genes, which can be used to quantify the expression of the normal albumin gene, using the abnormal albumin gene as an internal control (Ogawa et al., 1993). This method is applicable to hepatocytes and other cell types, such as bone marrow cells (Arikura et al., 2004; Inagaki et al., 2011).

The functionality of the transplanted F344 hepatocytes is evaluated based on the increased serum albumin levels in the recipients. Because the serum albumin level in F344-alb rats is extremely low, a small increase in albumin is detectable by sensitive methods such as the enzyme-linked immunosorbent assay (ELISA). However, conventional gel-electrophoretic

assays are unsuitable because the serum of F344-alb rats contains proteins with molecular weights similar to that of albumin, and the levels of other proteins may increase to compensate for the lack of albumin (Ohta et al., 1993b). Therefore, conventional gel electrophoretic assays may falsely detect protein levels that are much higher than those of the actual albumin levels.

### 5. Changeability of phenotype of hepatocytes *in vivo* and *in vitro*

Cultured hepatocytes differ from hepatocytes *in vivo* with respect to many properties. They often lose specific functions such as the production of albumin and the activity of tyrosine aminotransferase and cytochrome P-450, and gain bile duct epithelium-specific functions such as cytokeratin 19 expression (Block et al., 1996). This difference may arise because gene expression in hepatocytes is strongly influenced by the culture environment, which may activate specific transcription factors (e.g. AP1 and NFκB). In addition, the signals mediated by the extracellular matrix influence gene expression in hepatocytes *in vitro* (Serandour et al., 2005; Fasset et al., 2006; Kim et al., 2003). We studied whether the altered phenotype of cultured hepatocytes reverts to that *in vivo* when the cells are reimplanted into the body (Nishikawa et al., 1994). To investigate this problem, two markers were used, one of which is newly expressed and the other is suppressed in cultured hepatocytes, by the F344/ F344-alb transplantation model.

F344 hepatocytes were cultured on hydrophobic plastic dishes to form spheroidal aggregates. Within 3 days of culture, the hepatocytes formed spheroidal aggregates of approximately 50 to 100 μm in diameter, most of which were detached from the bottom and floated freely in the medium. After 5 days of culture, the spheroidal hepatocyte aggregates were harvested and implanted into livers and spleens of F344-alb. The hepatocytes in the liver tissue of F344 rats are positive for p450 (CYP2C6), which is extensively expressed in normal rat hepatocytes (Figure 2C), as well as albumin (Figure 2A), but these cells were completely negative for the placental form of glutathione S-transferase (GST-P), which is one of the glutathione S-transferases that plays an active role in the detoxification of xenobiotics and noxious products generated after tissue damage (Sato, 1999) (Figure 4B). Five days after the start of culture, although albumin expression was maintained in the hepatocytes in spheroidal aggregates (Figure 2D), GST-P was strongly positive in the nucleus (Figure 2E), but P-450 was completely negative (Figure 2F).

After intrahepatic transplantation, the transplanted hepatocytes could be identified by albumin staining within the recipient livers (Figure 2G). On day 5-10 after transplantation, most of the transplanted F344 hepatocytes were located at the portal veins. These cells were firmly attached to their walls and covered by endothelial cells, while some were occasionally observed integrated into the interlobular connective tissue. Most transplanted hepatocytes became completely negative for GST-P staining (Figure 2H), while P-450 was detected in all of the transplanted hepatocytes at an expression level equivalent to that of the surrounding host hepatocytes (Figure 2I). After intrasplenic transplantation, most hepatocytes migrated into the red pulps on days 5-10. These hepatocytes were stained positive for albumin (Figure 2J), still weakly positive for GST-P (Figure 2K) and strongly positive for P-450 which generally gave a more intense signal than in the hepatocytes of F344 livers (Figure 2L).

These results indicated that the phenotype of cultured hepatocytes returned to that of hepatocytes *in vivo* after implantation into intrahepatic and intrasplenic environments.

However, GST-P expression in the cultured hepatocytes was more rapidly extinguished when these cells were transplanted into the liver than into the spleen, and P-450 was more intensely expressed in the spleen than the liver, possibly as a result of differences in the environmental factors between these two organs. Although hepatocyte transplantation would appear possible to various organs other than the liver, the liver is considered to the most likely site for transplanted hepatocytes to return to physiologic functioning.

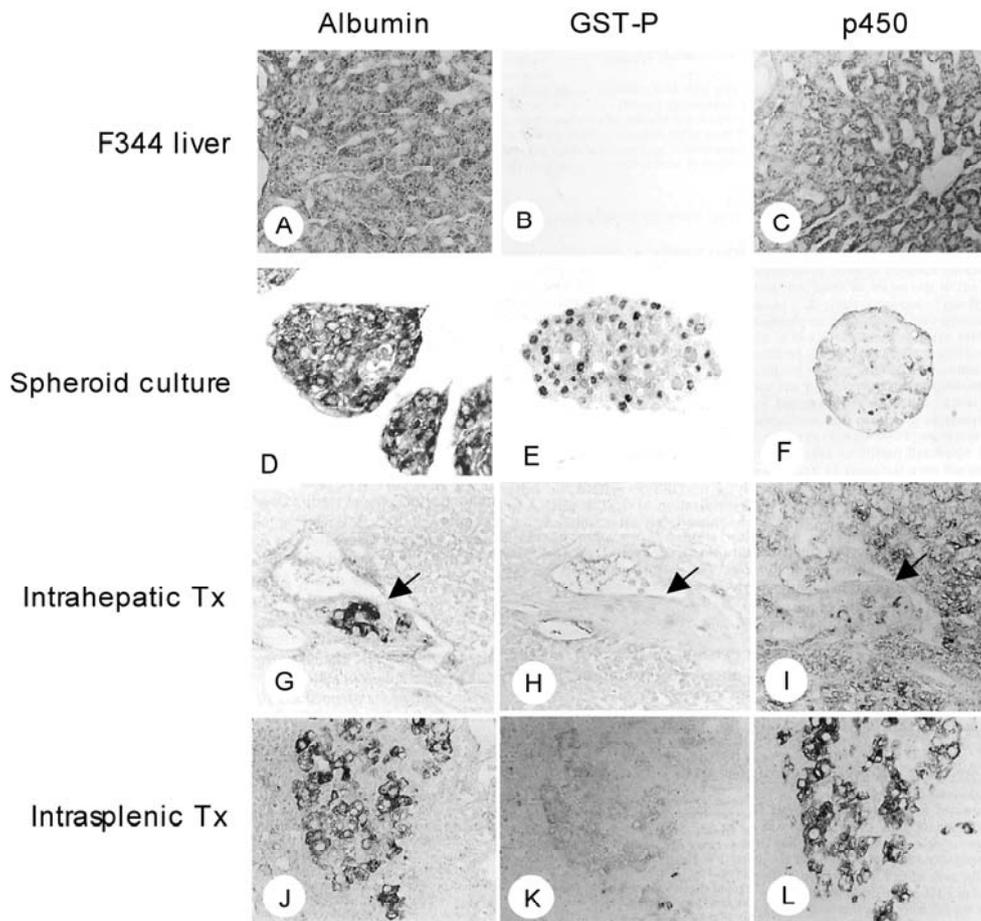


Fig. 2. Immunohistochemical staining for albumin, GST-P and P-450 (CYP2C6) in the hepatic tissue of F344 rat (A, B and C), spheroidal aggregates of F344 hepatocytes 5 days after the start of culture (D, E and F), and transplants of the cultured F344 hepatocytes in the liver (arrows in G, H and I) and spleen of F344-alb rats (J, K and L). A, D, G and J: albumin staining; B, E, H and K: GST-P staining; C, F, I and L: P-450 (CYP2C6) staining.

## 6. The effect of a continuous increase of serum albumin in analbuminemic rats

Albumin plays an important role in the maintenance of plasma osmotic pressure, and assists in the transport of various substances such as hormones, fatty acids, bilirubin, iron and other metals, and exogenous drugs. In spite of the absence of albumin, however, analbuminemic rats are generally healthy, showing none of the anticipated signs of disturbed physiology, such as edema and jaundice. This may be so fit because other serum proteins take the place of albumin. In analbuminemic rats, the total serum protein is usually normal because of increases in other serum proteins (Nagase et al., 1979). As the degree of increase is not uniform for all proteins, the electrophoretic pattern of the non-albumin fractions is quite different from that of normal rats. The major elevated components involved are  $\alpha$ 1-trypsin,  $\alpha$ 2-macroglobulin,  $\alpha$ -X protein, transferrin, ceruloplasmin, fibrinogen and immunoglobulins.

We investigated whether a continuous increase of serum albumin can normalize the unique patterns of serum proteins in analbuminemic rats (Ohta et al., 1993b). When carcinogen-induced preneoplastic hepatocytes isolated from hyperplastic hepatic nodules (HPN) of F344 rats were transplanted into F344-alb livers (Figure 3A), they could be induced to grow rapidly by application of the Solt and Farber regimen; sometimes these nodules progressed into hepatocellular carcinoma (HCC) (Ohta et al., 1994). As HPN and HCC cells can produce albumin and other serum proteins, this procedure allows the investigation of changes in serum protein content in analbuminemic rats under the condition in continuous albumin elevation.

The livers of all the F344-alb rats that had received F344 HPN cells contained a large number of HPN cells at 6 weeks (Figure 3B). At 12 months after transplantation, some recipients had HCC in addition to HPN. The transplanted cells were estimated to occupy approximately 6.0±1.4% and 30.0±10.0% of the total liver mass of the recipients at 6 weeks and 12 months, respectively. Serum albumin in F344-alb rats with HPN cell transplantation reached 7.0±2.4 mg/ml at 6 weeks and 38.8±7.9 mg/ml at 12 months; the latter number was comparable to or even higher than the values seen in normal F344 rats (34.0 ±0.2 mg/dl).

Although total serum protein in untreated F344-alb rats (6.8±0.4 g/dl) was similar to that in the untreated F344 rats (6.7±0.3 g/dl), electrophoresis of the serum proteins of the untreated F344-alb rats revealed that the  $\alpha$ ,  $\beta$  and  $\gamma$  fractions were elevated (Figure 3C a, b). On the other hand, although total serum protein in recipient F344-alb rats was almost at the normal level (7.0±0.4 g/dl) 6 weeks after transplantation, it was significantly higher at 12 months (10.4±2.1 g/dl) in these rats than in untreated F344 or F344-alb rats. Electrophoresis of the serum of F344-alb rats with HPN cell transplantation showed that albumin was clearly increased in the transplanted F344-alb cases, but the patterns of non-albumin fractions were unchanged compared with untreated F344-alb rats (Figure 3C b, c).

These results demonstrated that after transplantation of F344 HPN cells into F344-alb rats, although albumin was persistently elevated, the F344-alb-specific pattern of non-albumin fractions remained and was sometimes accompanied by an increase in total serum protein. The serum albumin level is determined by its synthesis, secretion, distribution and degradation in normal animals. When an excess of serum albumin is achieved by intravenous infusion of albumin in normal rats, both degradation and urinary excretion were increased (Rothschild et al., 1988). In contrast, the half-life of infused albumin is greatly

extended in analbuminemic humans and rats, although the half-life of other plasma proteins is normal (Inoue, 1985). It is thus possible that the lack of albumin causes an involution of the ability to maintain a normal albumin levels in analbuminemic rats. Additionally, albumin produced by the transplanted HPN cells may accumulate because of slow degradation or impaired urinary excretion, causing hyperalbuminemia in some recipients.

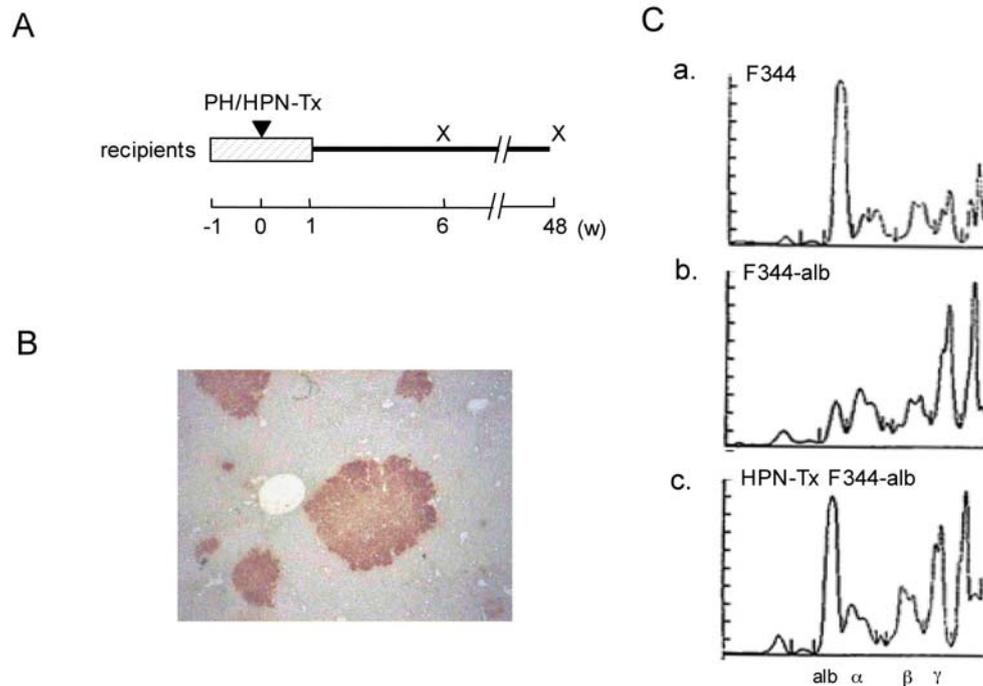


Fig. 3. The effect of continuous serum albumin elevation in F344-alb rats. A. Experimental procedure for transplantation of F344 HPN cells into the liver of F344-alb rats. Hatched box: dietary 2-acetylaminofluorene (2-AAF) treatment. PH/HPN-Tx: transplantation with HPN cells via the portal vein immediately after PH. X: sacrifice. B. Albumin immunostaining of a recipient F344-alb liver 6 weeks after F344 HPN cell transplantation. C. Densitometric analyses of electrophoretic patterns of serum proteins of untreated F344 and F344-alb rats, and as well as the F344-alb rats 12 months after HPN cell transplantation.

## 7. Hepatocytes derived from bone marrow cells

Hematopoietic cells contribute to the generation of hepatocytes in the liver. This process is thought mediated by the direct transdifferentiation of bone marrow cells into hepatocytes (Theise et al., 2000), the indirect transdifferentiation of bone marrow cells into oval cells that have the potential to give rise to mature hepatocytes during the cholangiocellular lineage (Oh et al., 2007), and the transfer of genetic materials from bone marrow cells to recipient hepatocytes by cell fusion (Wang et al., 2003). Using the analbuminemic rat model, we investigated whether bone marrow cells gave rise to hepatocytes during post-PH liver

regeneration (Arikura et al., 2004). In this process, the original hepatic mass is mainly regenerated by the division of resident hepatocytes.

The livers of one group of F344-alb rats were infused with F344 bone marrow cells via the portal vein immediately after PH (Figure 4A c). The bone marrow of another group of F344-alb rats were hematopoietically reconstituted using whole-body X-irradiation and F344 bone marrow cell transplantation with PH following 4 weeks later (Figure 4A d). Untreated F344-alb rats (Figure 4A a) and those treated with PH alone (Figure 4A b) served as controls. Four weeks after PH, although single cells or two-cell clusters of albumin-positive hepatocytes were seen regardless of bone marrow cell transplantation or PH status in both the control and experimental groups (Figure 4B a), clusters of more than 3 albumin-positive hepatocytes were observed in the livers of recipients that had undergone either bone marrow cell transplantation at the time of PH (Figure 4B b, c) or prior bone marrow reconstitution 4 weeks before PH. Normal albumin mRNA was detected in the RNA that was isolated from the livers of recipient F344-alb rats (Figure 4C a). Normal albumin gene sequences were also detected by PCR in DNA that was isolated from the micro-dissected albumin-positive hepatocyte clusters (Figure 4C b). In a female F344-alb rat that had been transplanted with male F344 bone marrow cells, albumin-positive hepatocyte clusters in the liver were positive for the Y chromosome marker *Sry3* (Figure 4D a, b).

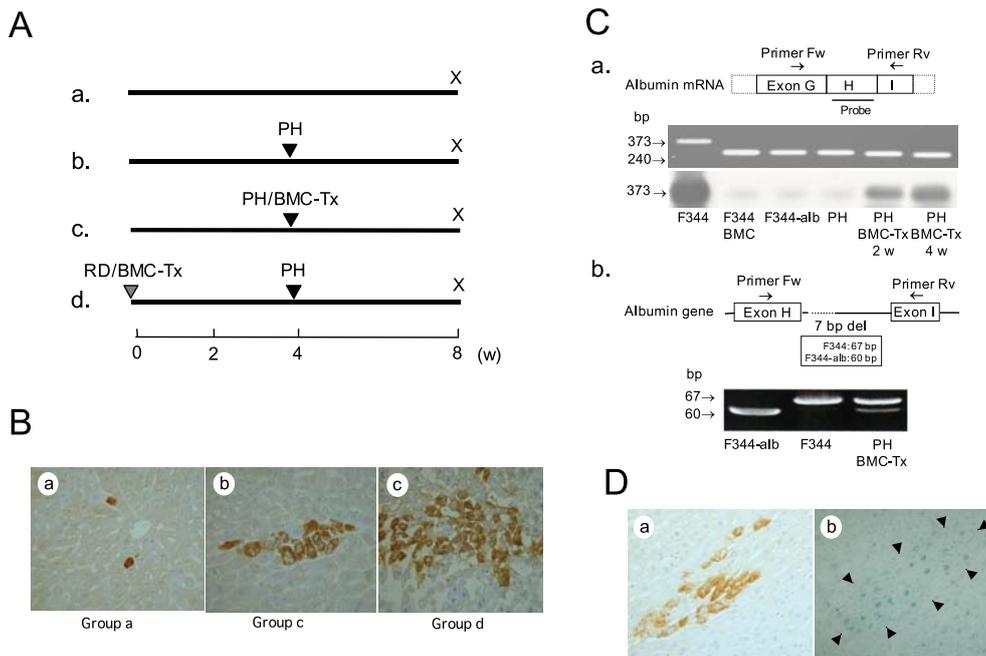


Fig. 4. Detection of bone marrow cell-derived hepatocytes in the F344-alb livers. A. Experimental groups: a. untreated F344-alb rats; b. F344-alb rats with PH alone; c. F344-alb rats with F344 bone marrow cell transplantation (BMC-Tx) immediately after PH; d. F344-alb rats with bone marrow reconstitution by whole-body X-irradiation (RD) and F344 BMC-Tx followed by PH four weeks later. B. Immunohistochemical staining of the hepatic

tissues for albumin; a. untreated F344-alb rats [Group a in (A)]; b. F344-alb rats with BMC-Tx immediately after PH [Group c in (A)] 2 weeks after PH/BMC-Tx.; c. The same group [Group c in (A)], 4 weeks after PH/BMTx.]. C. a. RT-PCR for albumin mRNA using the primers at exons G and I on RNA isolated from the liver and bone marrow of F344 and livers of untreated F344-alb, F344-alb with PH and F344-alb with PH plus BMC-Tx (2 and 4 weeks, Group c) (upper panel). Although only the exon H skipped albumin mRNA was amplified from the RNAs of control and transplanted F344-alb livers (middle panel), Southern blot analysis of the above PCR products using the exon H probe detected the normal albumin mRNA in the F344-alb livers with BMC-Tx (lower panel). b. PCR-based detection of normal albumin gene sequence from DNA isolated from an albumin positive hepatocyte cluster in F344-alb liver with RD plus BMC-Tx→PH (Group d) using the primers at exons H (forward) and I (reverse). D. *Sly3* *in situ* hybridization. a. Albumin immunostaining of female F344-alb liver with RD/male BMC-Tx→PH (Group d). b. *Sly3* *in situ* hybridization in the contiguous section of a.

We also investigated whether hematopoietic stem cells mobilized from the bone marrow into the peripheral blood can give rise to hepatocytes in the regenerating liver after PH (Huiling et al., 2004). The donor F344 rats were repeatedly treated with the recombinant granulocyte colony stimulating factor (G-CSF). Mononuclear cells were isolated from the peripheral blood and infused into the portal veins of F344-alb rats immediately after PH. Clusters of more than 3 albumin-positive hepatocytes and normal albumin mRNA and genomic DNA sequences were detected in the livers of recipient F344-alb rats.

These results demonstrate that F344 bone marrow cells can give rise to albumin-positive hepatocytes during liver regeneration after PH in F344-alb rats. However, the number of albumin-positive hepatocyte clusters was estimated to be 1000-2000 cells in the whole liver, accounting for 0.003-0.004% of the entire hepatic volume. Therefore, bone marrow cells play only a minor role in liver regeneration after PH. Interestingly, we detected large clusters of more than 50 albumin-positive hepatocytes; these clusters may consist of about 360 cells. Because hepatocytes divide one or two times after PH to restore the original hepatic mass, we hypothesized that some of the bone marrow-derived hepatocytes have a high proliferative capacity.

## 8. Protection from liver injury by bone marrow cell transplantation without liver repopulation

In the liver injury model in which the animals are pretreated with retrorsine (RS) followed by PH, transplanted hepatocytes repopulate the liver to replace a large hepatic mass with donor hepatocytes within 2-4 months (Laconi et al., 1998). However, bone marrow cell transplantation yields few or no donor bone marrow-derived hepatocytes in RS/PH treated animals (Wagers et al., 2002). Therefore, we investigated the effects of hepatocyte transplantation and bone marrow transplantation on RS/PH-treated livers using the analbuminemic rat model (Zhang et al., 2007).

F344-alb rats were given two doses of RS two weeks apart followed by PH in the 4th week (Figure 5A b). F344-alb rats that had received PH alone were used as controls (Figure 5A a). As expected, the RS/PH-treated rats showed a high mortality rate (survival rate 35%), with

death occurring between 1 and 11 days after PH, whereas all of the control animals survived (Figure 5B). Serum AST, ALT and bilirubin levels were elevated two days after PH (Figure 5C), and the liver tissue showed marked histological damage. In contrast, when hepatocytes (via the portal vein) or bone marrow cells (via the portal or penile vein) were transplanted immediately after PH in RS-treated F344-alb rats, the survival rate was significantly improved; the improvement was 50% for hepatocyte transplantation and 72.5% for bone marrow cell transplantation (Figure 5B). The liver function test results for these recipient F344-alb rats revealed that the AST, ALT and bilirubin values two days after PH were lower than those of the RS/PH-treated F344-alb rats without cell transplantation, and the levels in the recipient F344-alb rats were almost comparable to those of the untreated F344-alb rats or F344-alb rats with PH alone (Group a) (Figure 5C). Furthermore, the histological damage to the liver tissue was less than that seen in RS/PH-treated F344-alb liver tissue without cell transplantation.

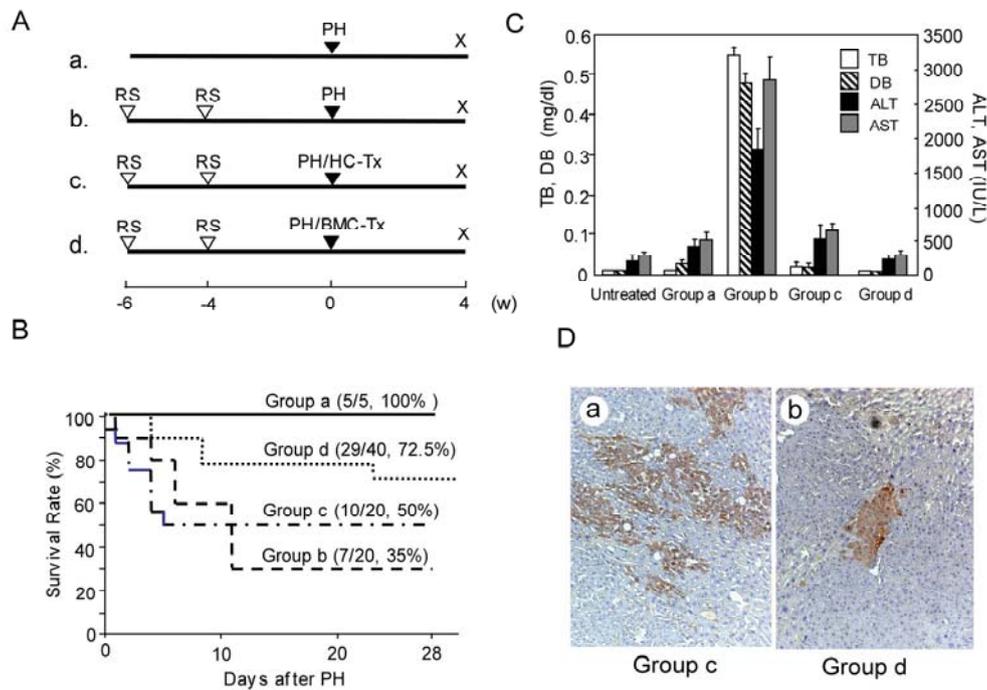


Fig. 5. The effects of hepatocyte transplantation (HC-Tx) and bone marrow cell transplantation (BMC-Tx) in the retrorsine (RS)/PH-induced hepatic injury model. A. Experimental groups: a. F344-alb rats with PH alone; b. F344-alb rats treated with two doses of RS (30 mg/kg of body weight) 2 weeks apart followed by PH two weeks after the second RS treatment; c. F344-alb rats treated with two doses of RS followed by treatment with PH and HC-Tx; d. F344-alb rats treated with two doses of RS followed by treatment with PH and BMC-Tx. B. Survival curve. Most RS/PH-treated F344-alb rats (Group b) died 1-11 days after PH, but the survival rate was significantly higher in the RS/PH-treated F344-alb rats given HC-Tx (Group c) or BMTx (Group d). The numbers in parentheses show survival vs. total numbers of F344-alb and their survival rate 28 days after PH, PH/HC-Tx or PH/BMC-

Tx. C. Liver function tests. TB: Total bilirubin, DB: direct bilirubin, ALT: alanine aminotransferase, AST: aspartate aminotransferase. D. Albumin immunostaining in the F344-alb livers. a. RS+PH/HC-Tx (Group c). b. RS+PH/BMC-Tx (Group d).

In the RS/PH-treated F344-alb rats that received hepatocyte transplantation (Group c), the liver tissue was extensively replaced by albumin-positive F344 hepatocytes (Figure 5D a), the serum albumin level was increased almost to the level of normal rats, and normal albumin mRNA was detected in the liver four weeks after PH. The liver regeneration rate in these rats was almost comparable to the rate in F344-alb rats that underwent PH alone. However, in the RS/PH-treated F344-alb rats treated with bone marrow cell transplantation (Group d), only small clusters of albumin-positive hepatocytes were detected (Figure 5D b). These results are similar to the results seen in rats treated with simultaneous PH and bone marrow transplantation or with bone marrow reconstruction followed by PH as described above (Figure 4B). Furthermore, the liver regeneration rate in the RS/PH-treated animals with bone marrow cell transplantation was as low as that in the RS/PH-treated F344-alb rats without cell transplantation.

Our results indicate that, although the bone marrow-derived hepatocytes do not extensively repopulate the RS/PH-treated liver, they can prevent RS/PH-induced liver injury and increase the potential for recipient survival. Factors derived from the bone marrow cells and hepatocytes may suppress RS/PH-induced liver injury, the mechanism of which remains elusive. Because it takes time for transplanted hepatocytes to repopulate the liver and restore hepatic functions, bone marrow cell transplantation may prevent recipient death from liver injuries that occurs during the period before the transplanted hepatocytes can repopulate the liver.

### **9. Induction of immunotolerance for allogeneic hepatocytes by donor bone marrow cell transplantation**

One of the main challenges that limit the efficiency of hepatocyte transplantation is the large cellular loss after transplantation. The majority of hepatocytes infused into the portal circulation are trapped within the hepatic vessels and cleared by granulocytes, macrophages and Kupffer cells. Therefore, only a small number of infused hepatocytes are integrated into the host hepatic tissue (Han et al., 2009; Okazaki et al., 2008). Moreover, the transplanted allogeneic hepatocytes activate the adaptive immune system of the recipients, thereby activating immunological mechanisms thus contribute to the elimination of these hepatocytes (Han et al., 2009; Okazaki et al., 2008; Bumgardner & Orosz, 2000). However, when host bone marrow is reconstituted using donor bone marrow cells, successful transplantations of allogeneic hepatocytes have been conducted between mouse strains expressing disparate major histocompatibility complex proteins (Vidal et al., 2008). For bone marrow reconstitution, intra-bone marrow bone marrow cell injection is more efficient than intravenous bone marrow cell transfusion (Ikehara, 2005).

We investigated the effect of hematopoietic reconstruction on allogeneic hepatocyte transplantation using Lewis (LEW) rats as donors and F344-alb rats as recipients (Inagaki et al., 2011); these types of rats express disparate major histocompatibility complex protein. As described above, when syngeneic F344 hepatocytes were transplanted into the liver of RS

plus PH F344-alb rats (Figure 6A a), the large hepatic mass was replaced by albumin-positive hepatocytes (Figure 6B a). In addition, the serum albumin levels were increased, and no inflammatory changes were observed in the liver four weeks after transplantation. In

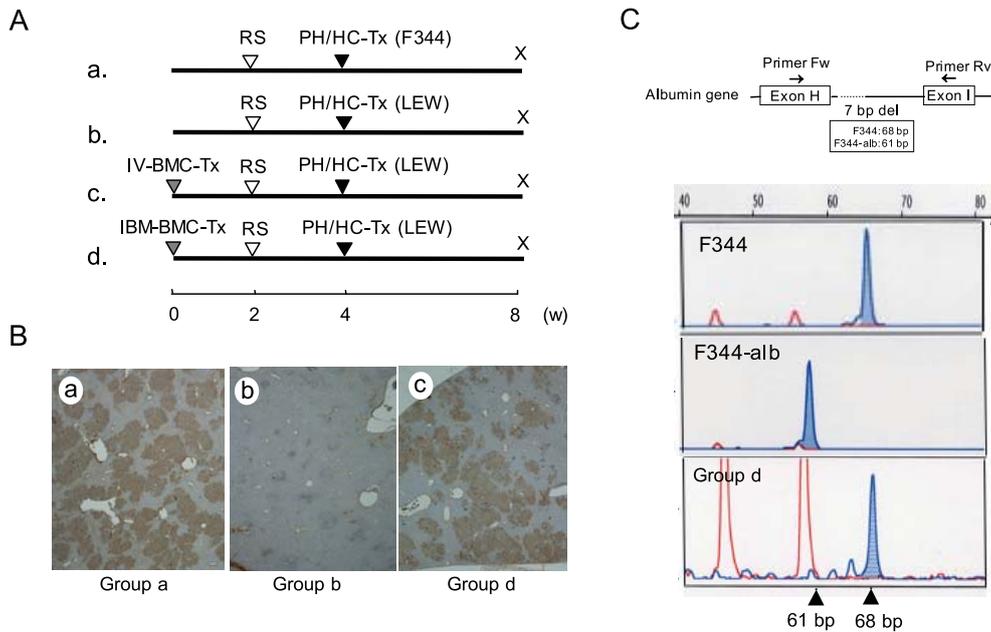


Fig. 6. The tolerance of allogeneic hepatocyte-mediated liver repopulation. A. Experimental groups: a: syngeneic F344 HC-Tx in RS/PH-treated F344-alb rats; b: allogeneic LEW HC-Tx in RS/PH-treated F344-alb rats; c: allogeneic LEW HC-Tx in RS/PH-treated F344-alb rats that received LEW BMC-Tx via intravenous transfusion (IV-BMC-Tx); d: allogeneic LEW HC-Tx in RS/PH-treated F344-alb rats that received LEW BMC-Tx by intra-bone marrow injection (IBM-BMC-Tx). B. Immunohistochemical staining for albumin in recipient F344-alb livers: a: F344-alb rats treated with syngeneic F344 HC-Tx [Group a in (A)]; b: F344-alb rats treated with allogeneic LEW HC-Tx [Group b in (A)]; c: F344-alb rats treated with allogeneic HC-Tx and prior IBM-BMC-Tx [Group d (A)]. C. PCR analysis of bone marrow reconstitution using the primers at albumin gene exons H and I and DNA isolated from the bone marrow cells of untreated F344 and F344-alb rats, as well as F344-alb rats treated with IBM-BMC-Tx [Group d (A)].

contrast, transplantation of allogeneic LEW hepatocytes into F344-alb rats (Figure 6A b) did not induce the repopulation of albumin-positive hepatocytes in recipient livers (Figure 6B b). In addition, serum albumin levels did not increase, and inflammatory cell infiltration was observed in the portal areas of recipient livers. When the bone marrow of the recipient F344-alb rats was reconstituted using LEW bone marrow cells (Figure 6A c, d), albumin-positive hepatocytes repopulated the livers of 1 of 6 F344-alb rats that underwent intravenous bone marrow cell transfusion and 6 of 6 F344-alb rats that underwent intra-bone marrow bone marrow cell injection (Figure 6B c). The latter was associated with increased serum albumin

levels. Although slight inflammatory cell infiltration was observed in the portal areas of the recipient livers, no inflammatory changes were detected in the areas repopulated with albumin-positive hepatocytes. When quantitative PCR was used to test bone marrow reconstitution in the recipient F344-alb rats for the normal and analbuminemic albumin gene sequences, 6 of 6 F344-alb rats showed bone marrow reconstitution after intra-bone marrow injection (Figure 6C). In contrast, only 1 of 6 F344-alb rats that underwent intravenous bone marrow cell transfusion showed bone marrow reconstitution. The albumin-positive hepatocyte repopulation and the increase in serum albumin levels depended completely on bone marrow reconstitution by the donor bone marrow cells.

These results indicate that liver repopulation via allogeneic hepatocyte transplantation without the use of immunosuppressants is possible if the recipient bone marrow is efficiently reconstituted using donor bone marrow cells. Intra-bone marrow injection of bone marrow cells induces bone marrow reconstruction in a manner that is more efficient than does intravenous transfusion.

## 10. Conclusion

Mito et al. (1978) completed the first successful hepatocyte transplantation in the rat spleen 33 years ago. Since then, considerable progress has been made in the field of hepatic cell transplantation research. Notably, it has been demonstrated that hepatocytes can be transplanted into the liver and can regenerate the diseased host liver. The use of hepatocyte transplantation is therefore expected to expand as a therapy for human liver disease. For cell transplantation to be used as a human therapy, further investigation is required to address number of problems including efficient ways to suppress the innate and acquired immune responses to the transplanted cells and the short- and long-term risks such as malignant transformation. The F344/F344-alb model provides a useful tool for studies of hepatocyte transplantation in the liver because it can be used to accurately trace the fate and functionality of the transplanted cells. Bone marrow cell transplantation may facilitate cell transplantation therapy via the hepatogenic potential of hematopoietic cells, the putative protective paracrine actions in the liver and the induction of donor-specific immuno-tolerance. A shift from orthotropic liver transplantation, if in part, to hepatocyte transplantation will yield new opportunities to develop therapies for human liver disease.

## 11. Acknowledgements

This work was supported by the grants from the Japanese Ministry of Science, Education, Sports and Culture.

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