

High Sensitivity of Neonatal Rat Hepatocytes to Retroviral-Mediated Gene Transfer and Their Transplantation into the Spleen of Adult Rat

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ABSTRACT. To optimize conditions for retroviral-mediated transfer of a recombinant gene to hepatocytes, the pMNSM-Tk-lacZ vector, which we had constructed to express the bacterial β -galactosidase gene, was transduced to rat hepatocytes under various conditions, and the expression of β -galactosidase activity was examined by cytochemical staining. Compared to the hepatocytes of adult rats, those of newborns were about 50–100 times more sensitive to transduction with the β -galactosidase gene *in vitro*. The sensitivity was high in the newborn hepatocytes when the virus was infected on days 1 and 2 after initiation of culture. However, the sensitivity to infection did not correlate with the DNA synthetic activity. The gene transfer was feasible not only to hepatocytes in monolayer culture but also to those in spheroid culture. The spheroidal aggregates containing hepatocytes transduced with the β -galactosidase gene could be transplanted into the spleen of syngenic adult rat, although the expression was very low.

Since the liver is subject to various inborn metabolic errors, there has been much interest in using genetically engineered cells for correction of the resulting diseases (14, 15). In order to restore the liver-specific functions by somatic gene therapy, hepatocytes are considered to be a suitable target for transduction of recombinant genes. This is because appropriate protein processing and the presence of cofactors and substrates are considered necessary for recombinant proteins to exert liver-specific physiological functions (3, 14–16).

It has recently been reported that of the various ways of transfection, the retroviral vector method is the most efficient to stably transfer recombinant genes to primarily isolated hepatocytes (17, 23, 29, 31). However, there are only a few reports in which the conditions for transduction, such as culture conditions, age of recipient cells, and timing of infection, have been investigated. In the present study, we compared the efficiency of transfection of hepatocytes between different ages of rats and between monolayer and spheroid culture using a retroviral vector containing the bacterial β -galactosidase (β -gal) gene. Furthermore, as hepatocytes can be transplanted into the spleen of syngenic rat (11, 21), and as the spheroidal aggregates of cultured hepatocytes are useful for implantation within the rat spleen

(25), we investigated whether it is feasible to transplant the β -gal-transduced hepatocytes into the spleen.

MATERIALS AND METHODS

Construction of pMNSM-Tk-lacZ. The *Hind*III-*Bam*HI fragment of thymidine kinase promoter/ β -gal gene cDNA coding sequences was isolated from the pLA plasmid (a gift from Dr. Kondoh, Dr. S. Noguchi, and N. Nakatsuji) and cloned to the *Hind*III-*Bam*HI site of pMNSM plasmid (28) (a gift from Dr. T. Tsuchiya), which contains the neomycin-resistant gene and simian virus 40 (SV40) promoter (Fig. 1). The recombinant plasmid, designated pMNSM-Tk-lacZ, was amplified by transfecting to *Escherichia coli* XL-1 Blue cells and purified by a Qiagen-pack 500 (Diagen) according to the manufacture's manual.

Production of recombinant retrovirus vector. The pMNSM-Tk-lacZ plasmid was transfected to the ecotropic packaging cells, Psi2 (19), by electroporation. After 48 h, the conditioned medium was harvested, filtered through a nitrocellulose filter (Millipore, 0.45 μ m pore size) and used to infect the amphotropic packaging cells, PA317 (20) (American Type Culture Collection No. CRL 9078). G418 (500 μ g/ml)-resistant clones from the PA317 cells were expanded and further subcloned. The virus-producing capacity of each clone was estimated by counting the number of G418-resistant clones after infection of NIH3T3 cells with the conditioned medium.

Culture and transfection of hepatocytes. The hepatocytes of adult (6-week-old male) and newborn (3-day-old male)

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Abbreviations used: β -gal, bacterial β -galactosidase; SV40, simian virus 40; X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactoside; BrdU, bromodeoxyuridine; LTR, long terminal repeat.

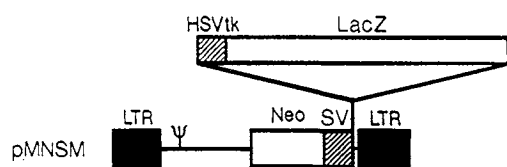


Fig. 1. Structure of the pMNSM-Tk-lacZ retrovirus vector. The *Hind*III-*Bam*HI fragment of thymidine kinase promoter (from -197 to +56)/ β -gal cDNA coding sequences was isolated from the pLA plasmid and cloned to the *Hind*III-*Bam*HI site of the pMNSM plasmid, which contains the neomycin-resistant gene and the SV40 early promoter. HSVtk, herpes simplex virus thymidine kinase promoter; LacZ, bacterial β -galactosidase gene; LTR, long terminal repeat; Neo, neomycin-resistant gene; SV, simian virus 40 early promoter.

F344 rats were isolated by collagenase perfusion via the portal vein according to the method of Berry and Friend (4) with a minor modification. In the case of adult rats, a two-thirds partial hepatectomy was performed 24 h before isolation of hepatocytes. For monolayer culture, the cells were plated onto 35 mm collagen-coated dishes (Corning) at a density of 2×10^5 cells in 1 ml of medium. On the other hand, the cells were plated for spheroid culture onto 52 mm hydrophobic dishes (Iuchi, Osaka, Japan) at a density of 4×10^5 cells in 2 ml of medium. The cells were cultured in Williams' medium E supplemented with 10% fetal calf serum for 24 h, and then in serum-free/hormonally defined Williams' medium E (6). The hepatocytes were infected using the conditioned medium of the virus-producing PA317 clone in the presence of Polybrene (8 μ g/ml) for 24 h.

Cytochemical staining for β -gal activity. The hepatocytes were fixed in 2% formaldehyde/0.2% glutaraldehyde solution for 5 min, washed in phosphate-buffered saline (PBS) 3 times, and incubated in the solution containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS. In the case of spheroid culture, the cell aggregates were pelleted after the cytochemical staining, dehydrated and embedded in paraffin. The thin sections were counter-stained in the nuclear fast red solution. The number of X-Gal-stained hepatocytes was counted under a microscope using an eyepiece disc. In the case of monolayer culture, the cell number in a dish was quantified by the fluorescence DNA assay (12) using hepatocytes cultured under the same conditions as above, and the frequency of X-Gal positive cells was expressed by percentage.

Assay for DNA synthesis. To label the DNA synthesizing cells, bromodeoxyuridine (BrdU) was added to the culture medium at a final concentration of 10 μ M for 1 h at various times after initiation of culture. The cells were washed in PBS and fixed in 70% ethanol for 30 min. The monolayer plates and the thin sections of spheroidal aggregates were sequentially treated with 2 N HCl and 0.1 M sodium tetraborate solutions. Then they were immunostained using the mouse biotin-streptavidin amplified system (Bio Genex). The labeling index of

the hepatocytes was determined by counting the number of labeled nuclei under a microscope.

Intrasplenic transplantation of β -gal gene-transfected hepatocytes. Neonatal hepatocytes were infected with the virus on the second day of spheroid culture, and the cell aggregates were harvested 24 h after infection. The aggregates were suspended in 0.2 ml Hanks' solution and infused into the spleen of adult rat using a 1 ml syringe with a 22 gauge needle (11, 21). During infusion, the splenic veins were clamped, and the spleen was loosely tightened at the lower portion immediately after the removal of the needle. The rats were sacrificed at various times after transplantation. The spleens were fixed in the 2% formaldehyde/0.2% glutaraldehyde solution for 1 h, sliced, and incubated in the solution for the X-Gal staining for 24 h at 30°C. The tissues were then fixed in Bouin's solution for 2 h and embedded in paraffin. Sections (4 μ m thick) were made and counterstained in the nuclear fast red solution.

RESULTS

pMNSM-Tk-lacZ vector. The β -gal activity was cytochemically demonstrated in almost all G418-resistant clones of the amphotropic virus-producing PA317 cells. Each clone was expanded and subcloned to establish the cells with high virus-producing capacity in the presence of G418. The viral titer of PA317 cells used in the following experiments was about 1×10^4 colony forming units/ml when the conditioned medium was infected to NIH3T3 cells.

Expression of β -gal gene in hepatocytes. After plating the adult hepatocytes to collagen-coated dishes, the cells spread to a monolayer within 3 h. On the other hand, when the cells were plated onto the hydrophobic dishes, the cells first loosely attached to the plates, then began to aggregate within days 1 and 2 and detached from the bottom of the plates, forming spheroidal aggregates during days 3 to 5. Although the adult hepatocytes were infected on various days after the start of culture, the X-Gal-stained hepatocytes were constantly very few in monolayer culture (0 to 40 cells/dish) (Figs. 2a, 3a), and absent in spheroid culture (Fig. 2b).

On the other hand, the shape and arrangement of newborn cells were almost the same in vitro as those of the adult cells, except that the newborn cells were smaller than the adult cells. The incidence of X-Gal-stained hepatocytes was much higher than in the adult hepatocytes (Fig. 2c). The sensitivity to infection was high when the cells were infected on days 1 and 2 (Fig. 3a). With newborn hepatocytes, the frequency of X-Gal-stained hepatocytes in monolayer culture was twice that in spheroid culture (Figs. 2d, 2e). When the hepatocytes were infected on days 1 and 2, the number of X-Gal-stained hepatocytes gradually decreased thereafter (Fig. 3b).

DNA synthesizing activity of hepatocytes. In the

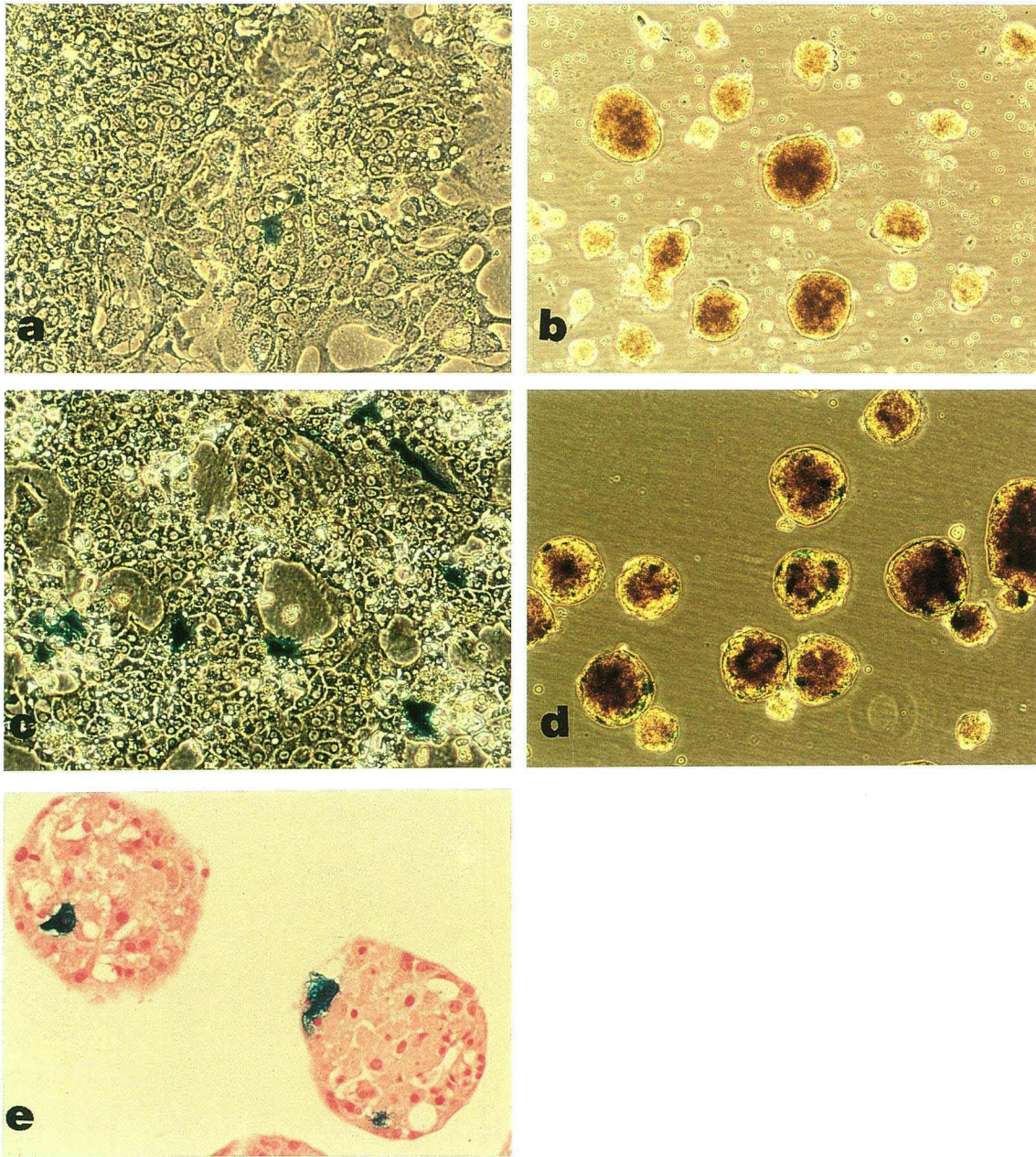


Fig. 2. Photos of X-Gal stained hepatocytes which were infected during day 2 and stained with X-Gal at day 4 after initiation of culture. a) Adult monolayer, b) adult spheroid (a and b, $\times 60$), c) newborn monolayer, d) newborn spheroid (c and d, $\times 50$), and e) thin section of spheroidal aggregates which contain β -gal-positive newborn hepatocytes ($\times 200$ Hematoxylin-Eosin).

case of adult cells, the labeling index most increased on day 1 in monolayer culture (Fig. 4). On the other hand, it elevated on day 3 in neonatal monolayer culture (Fig. 4). There was no large difference in maximum DNA synthesizing activity between adult and newborn cells under the conditions used. In spheroid culture, when the DNA synthesizing activity was examined on days 3 and 4, the labeling index was 4–5% in adult cells and 8–

10% in newborn cells.

X-Gal-stained hepatocytes in the spleen. The implants of hepatocytes were observed within the splenic parenchyma when the spheroidal aggregates of newborn hepatocytes (3 days after the start of culture) were infused into the spleens of 6 to 8 week-old rats. On days 1 and 2, the cells were mainly seen as clusters of various size within the splenic parenchyma. Although the cells

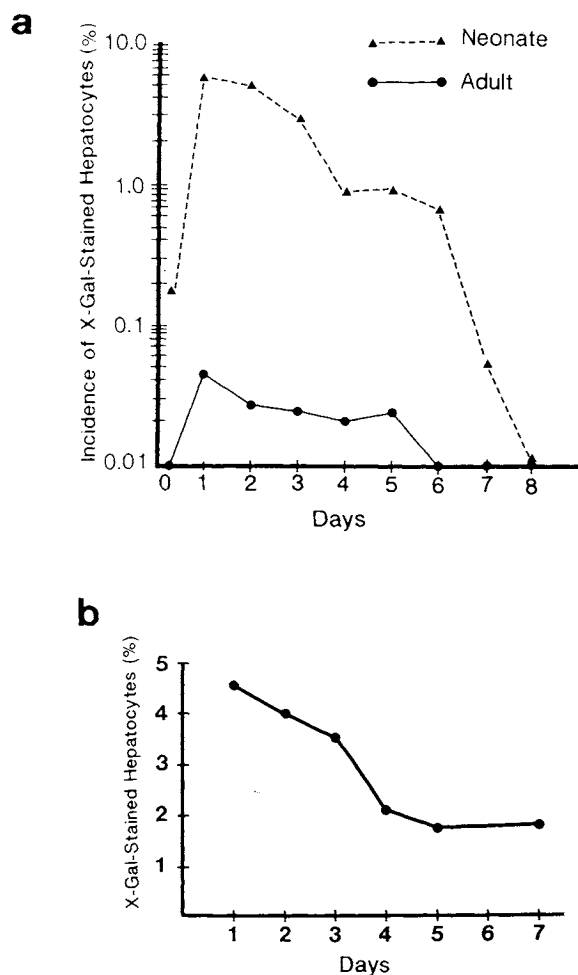


Fig. 3. The number of X-Gal-stained hepatocytes in monolayer culture. a) The cells were infected for 24 h with the conditioned medium of the PA317 cells and then cultured for 24 h in the normal medium. The values are the means of three separate examinations. Solid line: adult hepatocytes, dotted line: newborn hepatocytes. b) Typical change in the number of X-Gal-stained hepatocytes in neonatal monolayer culture. Hepatocytes were infected on day 1 and then maintained in serum-free/hormonally defined Williams' medium E. β -Gal staining was done on various days after infection. The abscissa shows days after the start of infection.

inside of clusters were necrotic, those at the peripheral areas were viable (Fig. 5a). On day 7, the hepatocytes were arranged in cords in the areas surrounding white pulp, and the number of necrotic cells had decreased. After X-Gal staining, occasional β -gal-positive hepatocytes were seen within the clusters of hepatocytes on day 1 (Fig. 5b). However, they were very few on day 2 and rarely seen on day 7.

DISCUSSION

Using the pMNSM-Tk-lacZ vector, the β -gal gene

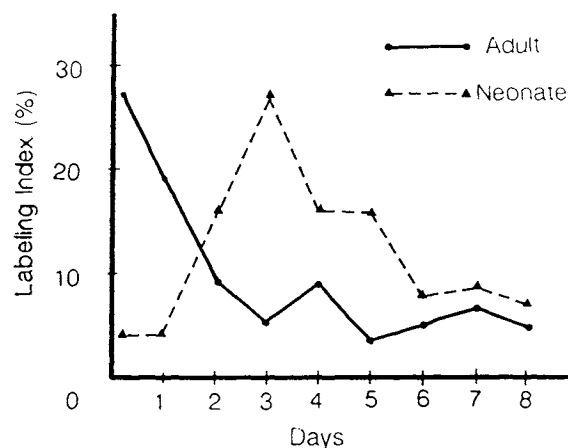


Fig. 4. BrdU labeling index of hepatocytes in monolayer culture. The cells were incubated with $10 \mu\text{M}$ BrdU for 1 h, fixed, and immunostained using anti-BrdU monoclonal antibody. The values are the means of three separate experiments. Solid line: adult hepatocytes; dotted line: newborn hepatocyte.

was successfully expressed in rat hepatocytes. As the β -gal gene is located downstream of SV40 and thymidine kinase promoters in this vector, it is considered to be

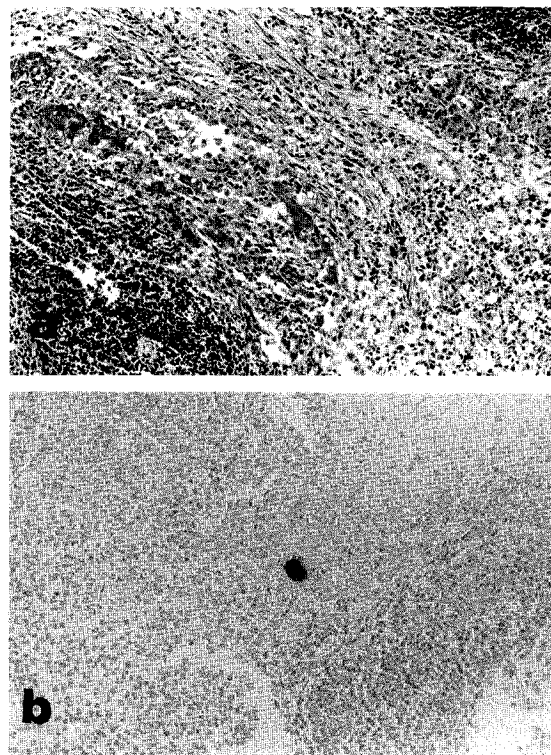


Fig. 5. Photos of transplanted hepatocytes expressing the β -gal gene in the spleen. a) Two days after transplantation (Hematoxylin-Eosin, $\times 100$). b) One day after transplantation (X-Gal staining hepatocyte, $\times 200$).

mainly controlled by the internal promoters rather than the long terminal repeat (LTR). Ledley *et al.* (17) reported that the retroviral vector which internally contains the thymidine kinase promoter was more effective for transduction of the gene in mouse hepatocytes than the vector without the internal promoter. Furthermore, Armentano *et al.* (3) reported that when rabbit hepatocytes were infected with a retroviral vector containing the internal cytomegalovirus promoter and human factor IX cDNA, 5 times more factor IX transcripts were produced from the internal promoter than from the LTR, whereas the same amount of the transcripts were produced from the two promoters in the packaging cells. So, it is conceivable that in hepatocytes, the presence of internal SV40/thymidine kinase promoter enhances expression of the recombinant gene.

Compared to adult hepatocytes, those of newborn rats were much more sensitive to transduction of the retroviral vector. It has been shown that the expression of viral receptors on the cell surface is the principal determinant for the susceptibility to infection with retroviruses and their derived vectors (24). Recently it has been demonstrated that human cells which have been transfected with cDNA of the murine ecotropic retroviral receptor gene acquire susceptibility to infection with murine ecotropic retroviral vectors (1). On the other hand, murine leukemia viruses were shown *in vivo* to infect fetal liver cells, but not those of newborn, adult, or regenerating liver (8, 9, 26), suggesting that the expression of the receptors in hepatocytes is related to the developmental stage.

The cultivation of hepatocytes seems to increase their sensitivity to infection, probably facilitating the expression of the retroviral receptors. The susceptibility increased on days 1 and 2 after the start of culture and then decreased in newborn hepatocytes. Wolff *et al.* (31) and Friedman *et al.* (7) had made similar observations in adult rat hepatocytes in monolayer culture when the cells were infected with other retroviral vectors. There was no clear relationship between the sensitivity to infection and proliferating activity either in adult or newborn hepatocytes. On the other hand, as liver-specific functions have been shown to decrease transiently after the start of monolayer culture in adult rat (18), the increased sensitivity has been considered to be related to the de-differentiation of the hepatocytes during this period (7, 31). The high sensitivity to infection in neonatal hepatocytes is consistent with a putative inverse relationship between transducibility and differentiation because neonatal hepatocytes are immature with respect to various liver-specific functions (22). The lower efficiency in spheroid culture may also be related to differentiation because spheroid culture is reported to preserve more liver-specific functions *in vitro* (10, 13).

Although primarily isolated hepatocytes can be trans-

planted into various parts of the body, there have been only a few reports concerning the transplantation of gene-engineered cultured hepatocytes (2). For transplantation of cultured hepatocytes, Demetriou *et al.* (5) and Thompson *et al.* (27) attached the hepatocytes to plastic micro-beads or fibers coated with collagen and heparin binding growth factor 1 *in vitro*, and then transplanted them within the peritoneal cavity. On the other hand, Saito *et al.* (25) produced spheroidal aggregates of hepatocytes and implanted them within the spleen of syngenic rat. As spheroidal aggregates can easily be formed by using hydrophobic dishes, and as newborn hepatocytes are highly sensitive to infection with retroviral vectors as observed in the present study, the method described here will provide a useful model for the somatic gene therapy of liver diseases.

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