ORIGINAL ARTICLE

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Human amniotic epithelial cells are promising transgene carriers for allogeneic cell transplantation into liver

Abstract As human amniotic epithelial tissue is formed on about the eighth day after fertilization, human amniotic epithelial cells (hAEC) may have multipotency to differentiate into various organs, such as brain, heart, or liver. In this study, we showed evidence of the synthesis and excretion of albumin by hAEC, by immunostaining and enzyme-linked immunoassay. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot analyses revealed the expression of albumin mRNA and protein, respectively. In addition, hAEC also demonstrated immunoreactivity to genetic markers of liver lineage, such as human serum albumin and α-fetoprotein. Transplanted hAEC to Scid mouse liver showed positive immunoreactivity to albumin and α-fetoprotein. Genetically modified cells containing the β-galactosidase (*LacZ*) gene (AxCALacZ) were integrated in liver parenchyma. Human polymorphic gene analysis in Scid mouse liver after the implantation of hAEC showed that these Scid mouse livers obviously contained this human-specific gene until day 7 after the cell transplantation. As hAEC do not cause any acute rejection by allotransplantation, we conclude that hAEC may be useful as a transgene carrier to treat patients with inherited liver diseases.

Key words Human amniotic epithelial cells · Cell transplantation · Albumin · Gene therapy · Liver stem cells

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Introduction

Liver cell transplantation has been advocated for gene therapy or acute liver support (Sigal et al. 1992). The survival and function of the transplanted hepatocytes has been demonstrated in mutant rats lacking bilirubin-uridine diphosphate glucuronosyltransferase activity (Gunn rat) and rats with inherited lack of plasma albumin (Nagase analbuminemia rat) (Demetrious et al. 1986; Gupta et al. 1991). The concept of a liver stem cell has been confirmed by studies on embryogenesis of the liver, on experimental carcinogenesis, and on the properties of non-hepatocytic epithelial cells (Grisham and Thorgeirsson 1997). The availability of these hepatic stem cells may accelerate the development of clinical liver cell transplantation.

On about the eighth day after fertilization, human amniotic epithelial cells (hAEC) are formed from amnioblasts, which are adjacent to cytotrophoblasts and line the amniotic cavity together with the rest of the epiblast. Accordingly, hAEC may have the potentiality to differentiate into various organs, such as the brain, heart, or liver. Immunologically, hAEC are naïve because they lack the expression of MHC class II and mildly express MHC class I (Sakuragawa et al. 1995). Previously, we reported clinical and biochemical benefits gained by the use of human amniotic epithelial tissue transplantation to patients with some lysosomal storage diseases (Sakuragawa et al. 1992). In addition, using immunocytochemical staining, we found that hAEC expressed markers for both neural and glial cells (Sakuragawa et al. 1996). Recently we reported that hAEC express choline acetyltransferase (ChAT) mRNA and ChAT protein, and actively synthesize and release acetylcholine (Sakuragawa et al. 1997). Moreover, we discovered the presence, synthesis, and release of catecholamines (CA) by hAEC (Elwan and Sakuragawa 1997) and monkey amniotic epithelial cells (Elwan et al. 1998) as indicated by high-performance liquid chromatography-electrochemical detection (HPLC-ECD) studies.

The present study was designed to provide evidence for albumin synthesis and excretion by hAEC. In addition, we

carried out a transplantation experiment using genetically modified hAEC transplanted through the portal vein of the Scid mouse as a trial for future clinical applications to gene therapy using hAEC as a gene carrier.

Methods

Preparation of cells

Human amniotic membrane was mechanically peeled from the chorion of a placenta obtained from a woman with an uncomplicated cesarean section and was extensively scraped out to remove the underlying tissues (the spongy and fibroblast layers) to obtain pure epithelial layer with basement membrane. This membrane was used to obtained hAEC, as previously described (Sakuragawa et al. 1995). The obtained hAEC were cultured in 100-mm plastic dishes in RPMI-1640 medium supplemented with 10% fetal calf serum, streptomycin 100μg/ml, penicillin 100U/ml, and glutamine 0.3mg/ml and incubated under a humidified atmosphere of 5% CO_2 in air at 37°C.

Immunostaining of cultured hAEC

Immunostaining, using hAEC grown on coverslips, was performed essentially by the avidin-biotinylated peroxidase complex method, as described before (Sakuragawa et al. 1996). The following primary antibodies were used: rabbit polyclonal antibodies (pAb) to human serum albumin (1:2,000 and 1:3,000) and α-fetoprotein (1:2,000; Chemicon International, Temecula CA, USA).

Western blotting

For Western blotting, cells were homogenized in 500μl of 50mM Tris-HCl buffer (pH 7.4) containing 1mM ethylenediamine tetraacetic acid (EDTA), 1mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100. The homogenate was centrifuged at 13,000rpm for 60min. The supernatant was lysed in sodium dodecylsulfate (SDS) buffer and boiled for 5min, and the sample was subjected to electrophoresis on a 7.5% polyacrylamide-SDS gel, using a protein mini-apparatus (Bio-Rad, Hercules, CA) at 200V for 30min. The gel was electrically transferred onto a nitrocellulose filter using a Trans-Blot system (Bio-Rad). The filter was blocked and then incubated overnight at 4°C with rabbit anti-albumin antibody, followed by three washes with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). Then the filter was incubated with horseradish peroxidase (HRP)-anti-rabbit IgG for 1h, followed by several washings with PBS-T, and detection with an (Amersham, Piscataway, NJ). Human liver cells and tissues were used as positive controls for albumin.

Reverse transcription-polymerase chain reaction (RT-PCR)

For the RT-PCR studies, total RNA was extracted by the acid guanidine-phenol-chloroform method. RT was performed with Super Script II RT (GIBCO BRL, Grand Island, NY) with a specific primer for human serum albumin (5′-CATCAACCTCTGGTCT GACC-3′) and α-fetoprotein (5′-ATGGGCCAC ATCCAGGACTA-3′). The cDNA product was then amplified for 35 cycles with forward and reverse primers using recombinant Taq DNA polymerase (Takara, Tokyo, Japan) and a thermal cycler. The amplification reaction was performed using a step wise program (94°C, 1min; 60°C, 1min; 72°C, 1min), followed by final extension at 72°C for 10min. The forward primers were 5′-CGTCG AGATGCACACAAGAG-3′ for albumin and 5′-ACTCCAGCATCGATCCC ACT-3′ for α-fetoprotein. The reverse primers were 5′-CAG CAGTCAG CCATTTCACC-3′ for albumin and 5′-TGGCTTGGAAAGTTCGGGTC-3′ for α-fetoprotein. Amplified PCR fragments were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized by UV illumination.

Detection of albumin in culture medium

The concentration of human serum albumin was determined by a two-antibody immunoassay, as outlined elsewhere (Harlow and Lane 1988). A 96-well plastic plate (Falcon 3915; Becton-Dickinson, Franklin Lakes, NJ, USA) was coated with anti-human albumin goat serum (no. 55109; Cappel, West Chester, PA, USA; 50μl/well, 200μg/ml of antiserum in PBS) at 4°C overnight. Control wells were coated with normal goat serum. After each well was washed twice with PBS containing 0.1% Tween 20, the sample, and standard albumin solution (human serum albumin [013-10501; Wako Pure Chemicals, Tokyo, Japan]) dissolved in RPMI-1640 containing 10% fetal calf serum [FCS]) were added to the wells and allowed to stand for 30min. The second antibody, HRP-conjugated anti-human albumin sheep IgG (AHP102P; Serotec, Oxford, UK) was dissolved in RPMI-1640 containing 10% FCS, to absorb cross-reactive antibodies to FCS. After incubation with the second antibody, PBS containing 3mg/ml o-phenylenediamine (P-8412; Sigma, St. Louis, MO, USA) and 0.03% hydrogen peroxide was added to each well. The detection limit of this system was statistically calculated to be 0.1ng/ml.

LacZ gene transfection into hAEC

Recombinant adenovirus containing the β-galactosidase (LacZ) gene (AxCALacZ) was used (a gift from Dr. I. Saito, University of Tokyo). Cells were exposed to AxCALacZ in a minimal volume at a multiplicity of infection (MOI) of 20. Two days after exposure to the virus, the cells were treated with 0.125% trypsin, then centrifuged at 1,000rpm for 10min. The cells were then resuspended in distilled water at a density of 5×10^5 cells/5μl.

Transplantation of hAEC to Scid mouse through portal vein

Scid mice (CB17; male, 10 to 15-weeks old) were purchased from Japan Clea (Tokyo, Japan). The Scid mouse received anti-asialo GM1 monoclonal antibody (MAb) (Wako Pure Chemicals) intravenously 1 day prior to cell transplantation. Cells were prepared at a concentration of 2.5×10^5/ml normal saline. The cell suspension (250 μl) was injected through the portal vein (with the animals under ether anaesthesia), using a 29-G needle syringe (Myjecter; Terumo, Tokyo, Japan). One to two weeks after the cell transplantation, the liver was removed and embedded in Tissue-Tek (Sakura Flnechemical, Tokyo, Japan) to obtain cryostat sections. Sections (2-μm-thick) were stained with either antibodies for human albumin and α-fetoprotein or X-gal for β-galactosidase. Prior to the immunohistochemical staining, HRP-conjugated anti-human albumin sheep antibody (AHP102P; Serotec) and anti-human α-fetoprotein goat antibody (AB635; Chemicon International) were injected to a non-treated Scid mouse, via the tail vein, to absorb cross-reactive antibodies to mouse proteins, and the antibody-containing mouse serum was used for immunostaining. Conjugation of anti-human α-fetoprotein with HRP was performed with periodate coupling (Harlow and Lane 1988). Cryosections were incubated with these pre-absorbed sera diluted 1:10 with PBS at room temperature for 1 h, followed by diaminobenzidine staining. β-galactosidase, a *lacZ* gene product, was detected by X-gal reaction, using X-gal (no. 027-07854; Wako Pure Chemicals) solution (4 mg/ml), containing 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, and N, N'-dimethylformamide. The sections were fixed briefly with neutralized formalin (10%) and incubated with X-gal solution at 37°C for 1 to 2 h.

All animal experiments were conducted according to the guidelines of the Animal Ethics Committee.

Human polymorphic gene analysis in Scid mouse liver

The human-specific gene was detected by PCR, using primers 5′-GAAACTGGCCTCCAAACACTGCCCGCCG-3′ (forward) and 5′-GTCT TGTTGGAGATGCACGT GCCCCTTGC-3′ (reverse). Total DNA was extracted from a Scid mouse liver block (approximately 1 mm³) by the phenol/chloroform method. The amplification reaction was performed using a step wise program (denaturing, 94°C, 1.5 min; annealing, 72°C, 2 min; extension, 72°C, 2 min) for 40 cycles. Amplified PCR products were subjected to electrophoresis as described above.

Results

Immunocytochemical staining of cultured hAEC

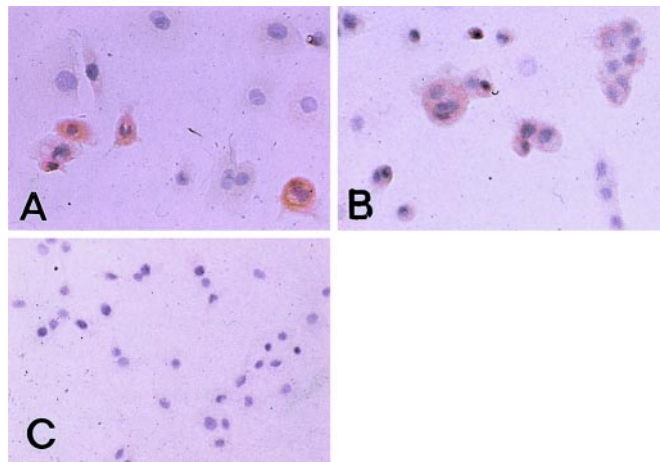The cultured cells (Fig. 1) revealed immunoreactivity to the polyclonal antibodies for albumin and α-fetoprotein. The

Fig. 1A–C. Immunostaining of human amniotic epithelial cells (hAEC) showed positive immunoreactivity to **A** polyclonal antibodies for human albumin and **B** α-fetoprotein. **C** Ig G control **A**, **B**, **C**, ×200
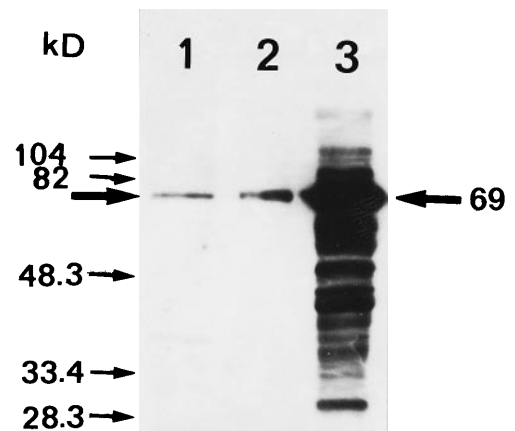

Fig. 2. Western blot analysis of human albumin in hAEC. *Lane 1*, hAEC; *lane 2*, human liver cells (ATCC CCL-13 Chang liver); *lane 3*, human liver tissue, obtained from an autopsy sample from an individual without liver disease. *Thick arrows* indicate the major band of 69 kDa

cultured cells showed a scattered distribution of positively and negatively stained cells, indicating heterogeneous expression of albumin and α-fetoprotein in hAEC.

Western blotting and reverse transcription-polymerase chain reaction (RT-PCR)

Western blotting using a polyclonal human albumin antibody showed a major band corresponding to the molecular weight of 69 kDa (Fig. 2). The targeted RT-PCR product, obtained from the tissue and primary cultured cells, suggested the presence of albumin mRNA in the hAEC. α-Fetoprotein mRNA was expressed in hAEC, but was not detected in human amniotic epithelial tissues (Fig. 3).

Synthesis of albumin by hAEC

Human albumin concentration in the culture supernatant of hAEC was determined by enzyme-linked immunoassay. A
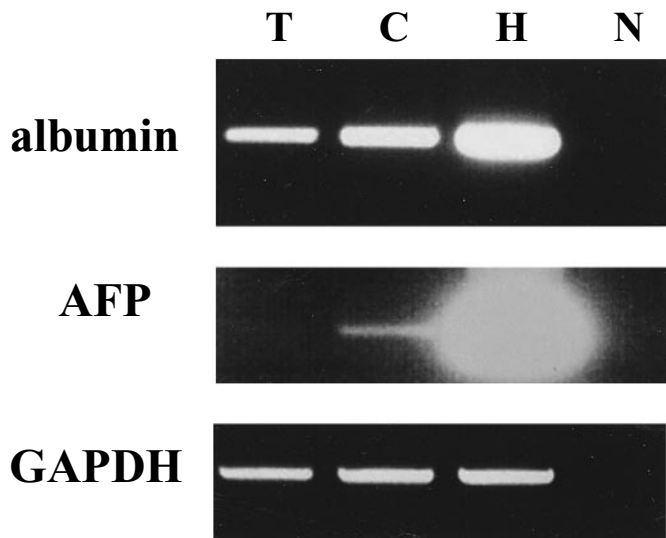
Fig. 3. Expression of human albumin and α-fetoprotein (*AFP*) mRNA in hAEC. *Lane 1*, Human amniotic epithelial tissue (*T*); *lane 2*, hAEC (*C*); *lane 3*, HepG2 cells as a positive control (*H*); *lane 4*, negative control (*N*). *GAPDH*

Fig. 4A–D. Immunostaining of Scid mouse liver 1 to 2 weeks after the transplantation of genetically modified cells via the portal vein showed cells with positive reactivion to **A** human serum albumin and **B** α-fetoprotein. **C**, **D** X-gal staining of Scid mouse liver 1 week after the transplantation of genetically modified cells via the portal vein showed a few blue cells in the parenchyma of liver. **A**, **B** ×400; **C** ×100; **D** ×400

significant level of human albumin was detectable from day 5 after the first passage of the cells. The concentration appeared to increase with culture time with the five samples obtained from five independent donors. Normally, hAEC grow under primary culture conditions and passages are performed once or twice a week. Albumin synthesis, however, was unlikely to start during the growing stage. Actually, albumin was detectable only from 5 days after the passage, ie, albumin was secreted after hAEC were in the fully confluent state. With the enzyme-linked immunoassay, albumin was detected in the cell incubation medium after 9 days of cultivation, with the medium containing 2.83 ± 2.05 ng/ml.

Transplantation of genetically modified hAEC into liver of Scid mouse

Immunostaining of Scid mouse liver 2 weeks after the injection of hAEC through the portal vein showed immunoreactive cells to antibodies for anti-human albumin (Fig. 4A) and anti-human α-fetoprotein (Fig. 4B). X-gal staining of liver 1 week after the transplantation of hAEC transfected with the *Lac Z* gene revealed several blue cells in the parenchyma of liver (Fig. 4C,D), indicating that the transplanted cells had been integrated into the hepatic parenchyma within a few days.

Human polymorphic gene analysis in Scid mouse liver after implantation of hAEC

The human gene-specific tandem repeat, D1S58 defined by the DNA probe for pMCT118, was detected after the hAEC transplantation (Fig. 5). Identical bands were 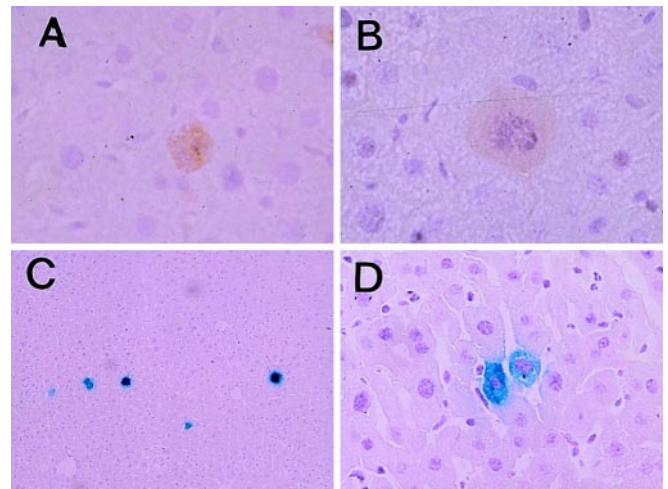amplified with liver transplanted with hAEC from the same donor, as the gene is inherited from both parental genomes and is polymorphic (Kasai et al. 1990). These Scid mouse livers obviously contained this human-specific gene until day 7 after the cell transplantation, whereas no PCR product was detected with DNA from normal Scid mouse under the present conditions.

Discussion

In the present study, immunostaining of cultured hAEC demonstrated the presence of immunoreactivities to albumin and α-fetoprotein. In addition to immunocytochemical staining, the present study utilized different techniques to demonstrate that hAEC possess native albumin synthesis activity. Western blotting showed the presence of albumin, while RT-PCR analysis revealed the expression of albumin mRNA by hAEC. Moreover, the enzyme-linked immunoassay provided further evidence for the presence of albumin and showed the capability of hAEC to synthesize and secrete albumin. Regarding the site of albumin expression, albumin can be visualized within the cytoplasm of liver cells by anti-albumin antibodies carrying a fluorescent or peroxidase label. Albumin mRNA was readily observed in liver, but no transcripts were found in other organs, such as kidney, brain, heart, lung, or intestine (Peters 1992). We found evidence of the synthesis and secretion of albumin in hAEC, although it was a small amount compared with findings in rat hepatocytes cultured in serum-free medium containing glucagon, insulin, and dexamethasone (Dahn et al. 1993). It is interesting to note that amniotic fluid contains about 2g/l of albumin (Bala et al. 1987). It is well known that the placenta is a highly selective organ in its transport of plasma proteins, including albumin. Labeled albumin

Fig. 5. Polymerase chain reaction (PCR) analysis of human polymorphic gene in Scid mouse liver. Livers were excised on the day indicated and total DNA was extracted. The human gene-specific tandem repeat, DIS58 defined by the DNA probe for pMCT 118. *Lane A*, Size marker; *lane B*, hAEC; *lane C*, normal Scid mouse liver; *lanes D–I*, Scid mouse liver transplanted with hAEC, 1 h (*D*), 1 day (*E*), 2 days (*F*), 3 days (*G*), 4 days (*H*), and 7 days (*I*) after the cell transplantation
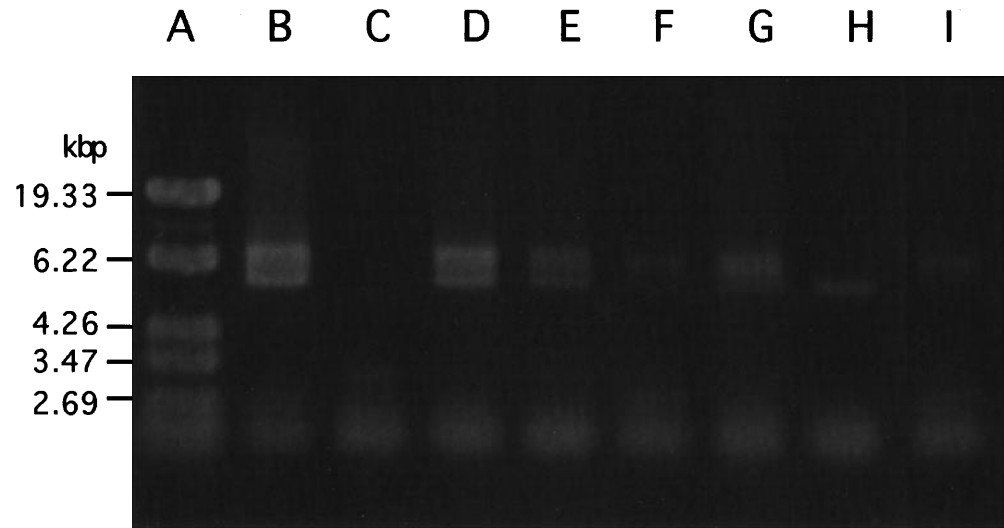
injected into a mother appears, in the fetus after 25 days, with no more than 5% of the maternal-specific activity (Gitlin et al. 1964). Further studies are now underway to elucidate whether amniotic epithelial cells may be a source of albumin in amniotic fluid.

Hepatocyte transplantation has been proposed for "gene therapy" for various types of inherited diseases expressed in the liver, and as treatment for acute liver failure of various causes. Indeed, hepatocyte transplantation has already been employed in a number of genetic liver diseases, including ornithine transcarbamylase deficiency (Reyers et al. 1996), familial hypercholesterolemia caused by a deficiency of low-density lipoprotein (LDL) receptors (Raper et al. 1996), and Crigler-Najjar syndrome type I (Fox et al. 1998). Experimentally, hepatocyte transplantation was successful in the Gunn rat and the Nagase analbuminemia rat. Xenogeneic hepatocellular transplantation provided functional LDL receptors that lowered serum cholesterol in the Watanabe heritable hyperlipidemic (WHHL) rabbit by 30%–60% for at least 100 days (Gunsalus et al. 1997). Acute liver failure is usually life-threatening, because of severe metabolic disturbance associated with multiple organ failure, hepatic coma, and encephalopathy. Because of the shortage of donor organs for orthotopic liver transplantation, several bridging techniques have been proposed to sustain patients until transplantation, including the temporary use of a xenogeneic organ (Starzl et al. 1993) and the use of a bioartificial liver (Strom et al. 1997). Lake (1998) suggested that the use of hepatocyte transplantation as treatment for a number of genetic and nongenetic diseases should be explored continuously, because there is a critical shortage of donor livers for patients with liver failure.

An attempt to use syngeneic fibroblasts for a transgene carrier was reported recently with the Gunn rat model; these genetically modified cells were shown to be effective in reducing unconjugated bilirubin level after transplantation into the peritoneal cavity (Seppen et al. 1997). However, Seppen et al. (1997) found that the fibroblasts formed a tumor 6 weeks after transplantation, suggesting that heterotopic localization of the physiological function may be undesirable. In this sense, hAEC will be more suitable as a transgene carrier to the liver than fibroblasts or other non-hepatic cells, as hAEC expressed mRNA of albumin and α-fetoprotein in addition to showing synthesis and excretion of albumin.

Current research strategies to develop hepatocyte transplantation for acute liver failure of any cause and to develop gene therapy for inherited metabolic disorders expressed in the liver are directed towards finding suitable sources of cells for grafting. Stem cells of human origin (if available), which can differentiate into hepatocytes when transplanted into the liver, would be ideal. From the present findings that hAEC possess native albumin synthesis capacity and express liver lineage markers and that these cells are immunologically naïve, we believe that these criteria may qualify hAEC to be considered as a possible candidate cell type for hepatocyte transplantation in acute liver diseases. Although transplanted cells were reported to survive and function in vivo for almost 1 month (Sakuragawa et al. 1992), we were able to plot the gene markers in vivo 2 weeks after transplantation. Genetically modified HAE cells are being used for experimental gene therapy in the Gunn rat and the WHHL rabbit, in studies which are now underway. In addition to hAEC being of human origin, non-tumorous, easily available, and in continuous unlimited supply, the use of hAEC does not seem to involve ethical problems, which do occur with orthotopic liver transplantation or xenogeneic transplantation.

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