Transplantation of Genetically Altered Hepatocytes Using Cell–Polymer Constructs

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MANY fatal metabolic liver diseases are caused by a single gene mutation. Insertion of the appropriate gene using retroviral technology may potentially treat these diseases.[1–3] We developed a model for hepatocyte transplantation using prevascularized cell–polymer constructs with hepatotrophic stimulation.[4,5] Our main objective in these studies was to transfect the human growth hormone (hGH) gene into rat hepatocytes and then demonstrate expression of this gene after transplantation in vivo. To accomplish these goals, we first set out to define the optimal conditions for in vitro gene transfer, gene expression in isolated rat hepatocytes, and cell harvesting prior to implantation. We then measured expression of the transfected gene within hepatocytes we had retransplanted into animal recipients using our polymer delivery system.

MATERIALS AND METHODS

Animals

Adult, male Lewis rats weighing 150 to 200 g (Charles River, Wilmington, Mass) were used as donors.

Hepatocyte Isolation and Culture Conditions

Hepatocytes were obtained using the collagenase perfusion method of Aiken et al[6] and purified using an 87% Percoll gradient. Hepatocytes were cultured in 75-cm^2 tissue culture flasks for in vitro gene transfer studies and in 175-cm^2 flasks for transplantation experiments. The flasks were coated with 1 μg/cm^2 type I collagen (Vitrogen, Celtix, Palo Alto, Calif) using the carbonate buffer adsorption technique.[7] Freshly isolated hepatocytes were plated at 10,000, 25,000, or 50,000 cells/cm^2 in chemically defined medium as previously described.[7]

Retroviral-Mediated Gene Transfer

Gene transfer was performed by incubating hepatocytes for 6 hours with an amphotropic retroviral supernatant containing either the MFG LacZ retroviral vector coding for β-galactosidase (β-gal) or the α-SGC-hGH retroviral vector coding for hGH. Different adjuvants (Aldrich, Milwaukee, Wisc) were tested, polybrene at 8 μg/mL and DEAE dextran at 16 and 32 μg/mL.

Analysis of Gene Transfer Efficiency

Transfection efficiency of β-gal was measured by cytochemical staining based upon the method of MacGregor and Caskey.[8] Cells expressing the β-gal gene product will stain blue in the presence of the chromogen 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (Sigma Chemical Co, St. Louis, Mo). Results were expressed as percent of positively stained cells.

The hGH levels were analyzed using a radioimmunoassay (RIA) kit from Nichols Institute Diagnostics (San Juan Capistrano, Calif). The tissue culture media was changed to DMEM for 6 hours and then removed and assayed for hGH. For in vivo analysis, serum hGH was quantitated every 2 days.

Southern Blot Analysis

DNA was isolated from cultured hepatocytes using a lysis solution (1% sodium dodecyl sulfate [SDS] and 500 μg/mL proteinase K in TNE [10 mmol/L hydroxymethyl amino methane, 1 mmol/L EDTA, and 100 mmol/L NaCl]). Following digestion, equal amounts of DNA and copy number standards were separated on a 1% agarose gel and transferred to nylon membrane. The membrane was hybridized with a ^{32}P-labeled LacZ probe in Quikhyb hybridization solution (Stratagene, La Jolla, Calif). Blots were washed and exposed to x-ray film.

Immunofluorescence Microscopy

After fixation with 4% paraformaldehyde and permeabilization with 0.1% Triton X-100 in 0.1 bovine serum albumin (BSA)/phosphate-buffered saline (PBS), a rabbit antibody against human hGH (UCB Bioproducts, Westbury, NY) was added to the cells at a 1:150 dilution for 60 minutes, followed by rhodamine-conjugated goat antirabbit immunoglobulin (Ig)G at 1:75 dilution. Intracellular hGH in cultured hepatocytes was seen.

Hepatocyte Harvest

Twenty-four hours after transfection, hepatocytes were removed from the plate by incubation in a saline buffer solution (0.01 mol/L HEPES, 0.15 mol/L NaCl, 0.007 mol/L KCl, and 0.005 mol/L $CaCl_2$) containing collagenase (Collagenase D, Boehringer Mannheim, Indianapolis, Ind) at 350 μg/mL for 45 minutes at 37°C. Detached hepatocytes were rinsed twice in William's E medium prior to implantation in vivo.

Cell Transplantation Into the Animal Recipient

A portacaval shunt was performed on inbred Lewis rats (Charles River) by ligation of the portal vein and creation of an end-to-side anastomosis with the vena cava. Seven days later, poly(vinyl alcohol) (PVA) sponges (1-cm diameter, 0.5-cm thick) (Unipoint Industries, High Point, NC) were implanted into the subcutaneous tissue. Additional polymers were implanted between mesenteric leaves 2 days later. Five days after polymer implantation and prevascularization, 10^7 cells in 0.5 mL were directly injected into the PVA polymer with a 20-gauge needle. Rat serum hGH levels were obtained before and after transfected hepatocyte transplantation. Cell polymer matrices were harvested at days 0, 3, 7, and 15 for histology and hematoxylin & eosin staining.

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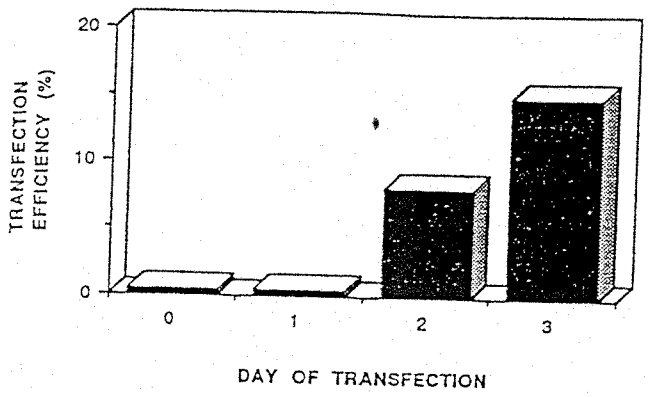

Fig 1. Transfection efficiency as a function of time at which the retroviral vector was added to the cultured hepatocytes, which were plated on day 0.

RESULTS

Optimizing Retroviral LacZ Gene Transfer in Cultured Hepatocytes

To determine the optimal conditions for gene transfection, we examined different variables. First, the time of transfection after hepatocyte plating was varied. We added retroviral supernatant with DEAE dextran (16 μg/mL) as an adjuvant to the cultures for 6 hours on days 0, 1, 2, or 3 after cell plating. Transfection efficiency (% cells transfected) increased from approximately 1% to 15%, with day 3 yielding the optimum efficiency (Fig 1). Then, we set out to define a cell density that can provide the maximum number of hepatocytes without sacrificing transfection efficiency. Three cell densities were tested: 10,000, 25,000, and 50,000 cells/cm^2. With the same conditions described above, transfection efficiency reached 20% as cell plating density was increased from 10,000 to 25,000 cells/cm^2 (Fig 2), but decreased at higher plating density (50,000 cells/cm^2), in which cells appear confluent. Adjuvants to gene transfer were also tested: polybrene at 8 μg/mL and DEAE dextran at 16 or 32 μg/mL. These studies revealed that the

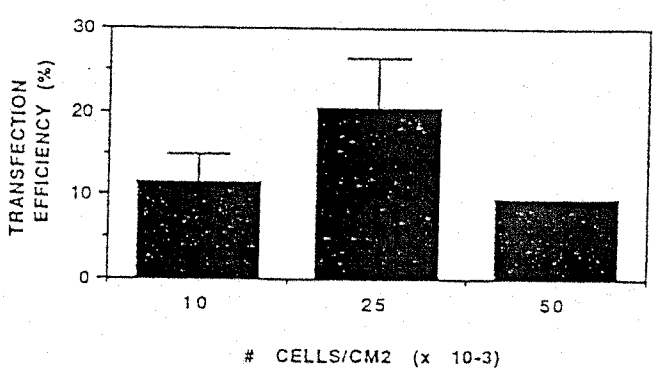

Fig 2. Transfection efficiency as a function of initial cell density plating. The means of three experiments are presented for cell densities of 10,000 and 25,000 cells/cm^2, while 50,000 cells/cm^2 were examined in only one experiment.

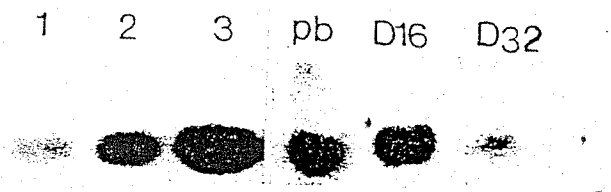

Fig 3. Southern blot analysis of proviral copy numbers. Lanes 1 to 3 represent 0.5, 1, and 2 copy numbers of standard LacZ-digested DNA, respectively. Copies in transfected hepatocytes with either polybrene (8 μg/mL), DEAE dextran at 16 mg/mL, or DEAE dextran at 32 μg/mL were estimated to be, respectively, 1, 1.5, and <1.

best transfection efficiency (approximately 16%) was obtained by adding DEAE dextran at 16 μg/mL to the retroviral supernatant (data not shown).

Southern analysis showed LacZ gene integration in cultured hepatocytes transfected at day 3, with either polybrene (8 μg/mL) or DEAE dextran (16 and 32 μg/mL). When compared with the copy number standards, approximately one to two copies per cell are seen in hepatocytes transfected with DEAE dextran at 16 μg/mL; less than one copy is estimated in hepatocytes transfected with polybrene or DEAE dextran at 32 μg/mL (Fig 3).

In Vitro Expression of hGH Gene

Once optimal in vitro conditions for retroviral gene transfer were defined, the vector SGC-hGH containing the gene for hGH was chosen for in vivo analysis. Using the conditions for optimal transfection described above, we detected hGH in the culture medium of transfected hepatocytes ranging from 0.5 to 1.5 ng/mL over a 6-hour period (data not shown). Successful transfection was also confirmed using an antibody against hGH. We detected positively stained perinuclear vesicles within cultured hGH-transfected hepatocytes.

Transplantation of Transfected Hepatocytes

Transfected hepatocytes were harvested from the tissue culture dish by using collagenase at different concentrations to obtain optimum cell viability prior to transplantation in vivo. An intermediate concentration of 375 μg/mL gave an optimum viability of 70%.

Histological examination of tissue sections from the cell–polymer construct was performed at different time points after hepatocyte implantation. Organized plates of viable hepatocytes were seen as late as 15 days after implantation filling the spaces of the prevascularized polymer device.

In two animals containing implants with the transfected cells, serum hGH levels, undetectable prior to cell injection, were quantitated at 0.275 and 0.440 ng/mL, respectively, on day 2 (Fig 4). Both these values are within the human physiological range (0.1 to 25 ng/mL). Subsequent serum samples at later time points were negative for hGH. Additional experiments have been performed that pro-

Fig 4. Transfected hepatocyte polymer constructs 7 days after cell implantation. Organized plates of hepatocytes (H) can be seen embedded in a fibrous vascularized tissue (F) within the spaces of the polymer sponge (P).

duced no detectable hGH following implantation of transfected hepatocytes. In each of these experiments, however, low transfection efficiency or low cell number following collagenase harvest was observed.

DISCUSSION

The liver is an important target for gene therapy because many inborn errors of metabolism are due to single gene mutations.[9,10] Methods for cell transplantation have been described using either splenic,[1] intraperitoneal, or portal vein injection[3] with limited success. Here, we describe a system for gene therapy using direct cell injection into prevascularized cell–polymer constructs.

Our optimization studies revealed that the hepatocyte culture conditions prior to retroviral gene transfer were important for maximal gene expression. We also found that to obtain a higher transfection efficiency a cell density below confluence (20,000 cells/cm^2) is necessary. Further, in accordance with the literature[3,12,13] we find that day 3 is the optimal time for gene transfer. This time is coincident with late S phase and the beginning of cell division.

The choice of adjuvant can greatly influence the efficiency of transfection. While most studies with hepatocytes use polybrene,[3,11,12,13] in our hands DEAE dextran at 16 μg/mL proved most effective. The reason for DEAE dextran's better performance is unclear, but it demonstrates the need to test adjuvants with each cell type and culture condition.

After in vitro optimization studies on retroviral-mediated gene transfer into adult rat hepatocytes, we demonstrated expression and secretion of a transfected gene product (hGH) into rat serum in two animals bearing cellular implant devices. Importantly, blood levels for hGH were found to be within physiological range (0.1 to 25 ng/mL) in two different animals. Given that the half-life of hGH is short (<20 minutes), it is likely that maintenance of levels for 2 days is due to survival and continued function of the implanted hepatocytes. Previous reports, using hGH-transfected myoblasts, demonstrated higher transfection efficiency and enhanced gene expression in the animal recipient, perhaps due to the greater proliferative capacity of myoblasts.[14,15] However, myoblasts may not be appropriate for restoration of liver function. Our goal is to develop a method using freshly isolated, nontransformed hepatocytes.

The strength of our system is reflected in the histology, which shows that hepatocytes engraft inside the prevascularized polymer construct and form organized cellular plates in cordlike structures reminiscent of the native liver (Fig 4). Further, infiltration of the polymer by mesenteric blood vessels, which enhances cell engraftment,[5] will facilitate delivery of the gene product into the systemic circulation.

In conclusion, we have begun to identify the optimal conditions for in vitro retroviral-mediated gene transfer into rat hepatocytes. Different animal models for gene therapy have already been described.[1,3,14,15] In this study, we demonstrate the feasibility of transplanting genetically altered hepatocytes utilizing prevascularized cell polymer constructs. The system studied and described above may have applications for in vivo delivery of genetically engineered hepatocytes for use in gene therapy.

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