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Graft-directed gene transfection in a rat model of liver transplantation

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ABSTRACT

In this model of liver transplantation, liver grafts were transfected with the E1-deleted, replication-defective adenovirus vectors encoding the LacZ gene driven by the human CMV promoter, or the replication-defective retrovirus vectors encoding the human IL-7 and neomycin phosphotransferase genes. Liver grafts were perfused ex-vivo with either of these two vectors during cold preservation time. In adenovirus-mediated gene transfer, positive staining of the hepatocyte was recognized predominantly around the portal triads at post-operative day 2. Positive staining was observed until 14 days after transfection. In a retrovirus-mediated gene transfection, gene transfection was tested both in a whole-size and a reduced-size graft implantation of the liver because the retrovirus vector requires a replication of the target cells for gene transfection. In conclusion, adenovirus-mediated gene transfection to the liver graft during cold preservation time resulted in an effective expression of the transfected sequence. On the other hand, retrovirus vector required the reduction of the liver graft for a successful gene transfection. *Ryukyu Med. J., 17(1)21~24, 1997*

Key words: gene transfer, adenovirus, retrovirus, liver transplantation

INTRODUCTION

Replication-defective retrovirus can be a shuttle vector for stable integration of proviral sequences in replicating cells, such as lymphocytes, tumor cells, and bone marrow cells[1-3]. Recently replication-defective adenovirus has also been increasingly used as a shuttle vector for gene transfer, because of their wide range of infectivity to the target cells, regardless of cell cycle, and high viral stock titers[4]. In the transplant setting, genetical modification of the graft organ may provide an alternative technique for either immunosuppression or graft preservation, in allogeneic or xenogeneic transplantation. In this study we have tested the feasibility of gene transfection of these two vectors in a model of liver transplantation in rats. In our previous report, adenovirus-mediated gene transfection to the heart graft was successfully performed with an ex-vivo perfusion of the heart graft[5]. Thus the same model of ex-vivo perfusion was applied to the liver graft in this study, under a cold preservation condition.

MATERIALS AND METHODS

Adeno-virus vectors

The adenovirus vector AdHCMVsplLacZ[6] contains an expression cassette encoding the *E. coli* β-galactosidase (β-gal), replacing the entire viral E1A region and most of the E1B region. Large scale production of adenovirus vectors was performed by infection (MOI 10:1) of 293 cells (density of $4x10^5$/ml) in SMEM suspension medium (Sigma), supplemented with 10% FCS (Sigma). Infected 293 cells were lysed after 48 h using 3 freeze-thawed cycles, pelleted at 2000 rpm for 10 min, and stored at -70℃. Viral titers were determined by plaque forming assay, and expressed as plaque forming units (pfu)/ml.

Retrovirus vectors

The JZEN hIL-7/thymidine kinase (tk) neo vector is a murine myeloproliferative sarcoma virus-based amphotropic vector (titer 10^7 colony-forming units/ml), in which the hIL-7 cDNA is driven by the 5' long terminal repeat (LTR) and the neoR gene by the tk promoter.

Animal model and orthotopic liver transplantation

Adult Wister rats, 10 to 16 weeks old, were used both as donors and recipients. Rat orthotopic liver transplantation was performed as described previously[7] under sterile conditions and light ether anesthesia. In brief, donor liver was harvested immediately after flushing in

situ with heparinized cold saline. Whole-size or reduced-size liver grafts were used depending on the protocol. Reduced-size grafts were prepared by resecting the left lateral and the caudate lobes, during a cold preservation. These grafts were further perfused ex-vivo with viral vectors and preserved at 4℃. Total cold ischemia time was adjusted to 60 min in every graft. In the recipient operation, the supra-hepatic vena cava was reconstructed with a full circumferential anastomosis, and the infra-hepatic vena cava and the portal vein were reconstructed using a cuff technique. The bile duct was reconstructed with a polyethylene tube. Each recipient was isolated in a private cage and administered antibiotics subcutaneously, consisting of tobramycin, 1 mg/day, for 2 consecutive days post transplant.

Experimental Design

Each graft were transfused either with $1x10^{10}$ pfu of AdHCMVsplLacZ in 2 ml of lactate ringer (LR) solution in group 1 or 2x108 cfu of JZEN vector in 2 ml of LR in group 2. In group 2, both the whole-size and the reduced-size grafts were used for a perfusion of viral vector. The perfused grafts were orthotopically implanted into syngeneic recipients and 3 recipients were each sacrificed at 2, 7 and 14 days after transplant. Tissue samples from the liver grafts were obtained from 3 different locations including the median lobe, left lateral lobe, and caudate lobe. In addition, serum and kidney samples were also collected at each sacrifice. All samples were stored in liquid nitrogen until use.

X-gal Staining

Eight micron thick frozen sections were produced on a cryostat, fixed with 1.25% glutaraldehyde at 4℃ for 10 min. After removing the glutaraldehyde, the preparations were immersed in 5-bromo-4-chloro-indolyl-β-D- galactopyranoside (X-gal) solution, and counter-stained with eosin. The cells expressing β-galactosidase turned blue in the presence of X-gal, while the non-expressing cells demonstrated either a clear or faintly yellow color. The stained sections were examined by light microscopy. The positively stained cells were counted in 10 high power fields (400X) in the liver.

Polymerase chain reaction (PCR) analyses

The genomic DNA was obtained from homogenized frozen sections using phenol-chloroform extraction followed by suspension in Tris-EDTA buffer and then was stored at −70 ℃. The positive control consisted of DNA extracted from AdHCMVsplLacZ-producing 293 cells. One microgram of DNA was amplified by PCR in a total volume of 50 μl which contained 0.2 mM deoxynucleoside triphosphatase, 0.25 mM 5' and 3' oligonucleotide primers, 50 mM KCL, 5 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, and 2.5 U Taq polymerase (Perkin-Elmer, Norwald, CT). PCR was performed in a DNA thermal cycler (Perkin-Elmer). The amplification profile consisted of 30 cycles of denaturing at 90℃ for 1 min, the annealing of primers at 60℃ for 2 min, and extension at 72℃ for 2 min. The primer sequences were chosen from separate exons of the genes in which the cDNA product detected the sequences of the *E. coli* β-gal actosidase or the human IL-7/neo R. The PCR products obtained in this manner were then analyzed by gel electrophoresis and southern blot.

O-Nitrophenyl-β-D-galactopyranoside (ONPG) Assay

To quantify the recombinant β-galactosidase production, an enzymatic assay was performed on liver protein. An aliquot of the total liver protein was incubated in ONPG buffer (0.1M $MgCl_2$, 4.5M β-mercaptoethanol, 4 mg / ml o-nitrophenyl- β -D-galactopyranoside, 0.1M Na_2PO_4) at 37℃ for 30 min. The reaction was then halted by the addition of 1M Na_2CO_3. The optical density of the solution was read at 420 nm, and compared to a standard curve generated with a known quantity of recombinant β-galactosidase. The results were then expressed as a percent β-galactosidase per total cellular protein.

RESULTS

Graft survival and light microscopic findings

Post-operative death related to technical failure occurred in less than 5 % of all recipients, and was excluded from the study analysis. The light microscopic study did not reveal any differences between the three groups. No specific inflammatory findings, which might be attributed to the adeno- or retro- viral infection, were observed in any of the liver grafts (Data not shown).

DNA PCR

Using PCR, DNA extracted from the liver graft or the kidney was evaluated for the presence of LacZ or IL-7/neoR sequences. All liver grafts demonstrated LacZ sequence until POD 14 in groups 1 (Fig. 1). PCR and southern blot analysis demonstrated hIL-7/neoR sequence until POD 21 in group 2 (Fig. 2). No animal showed a detectable band in the kidney in both groups.

ONPG assay

The quantity of viral-derived, recombinant β-gal protein in group 1 was determined by comparing its optical density with the optical density of purified *E. coli* β-gal, using ONPG assays. The levels of recombinant β-gal protein were 0.13±0.07% liver protein in group 1 and 0.007±0.003% in negative control (perfused with LR without vector), at POD 2. These levels in groups 1 decreased along with the post-operative course and had identical levels with negative control at POD 14 (0.008±0.006% liver protein).

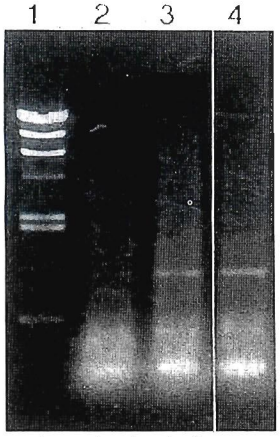

Fig. 1 In the samples obtained from group 1 animals, transfected gene sequence was examined by PCR using synthetic oligomers with sequences specific for E coli β-gal. The inserted β-gal gene (1036 bp) was detected in the viral packaging cell line (lane 4), and in the vector perfused liver grafts of group 1 at POD 7 (lane 3: perfused with $1x10^{10}$ pfu of AdHCMVsp1LacZ) but not in the control graft at POD2 (lane 2: perfused with LR). The DNA ladder marker was run in lane 1.

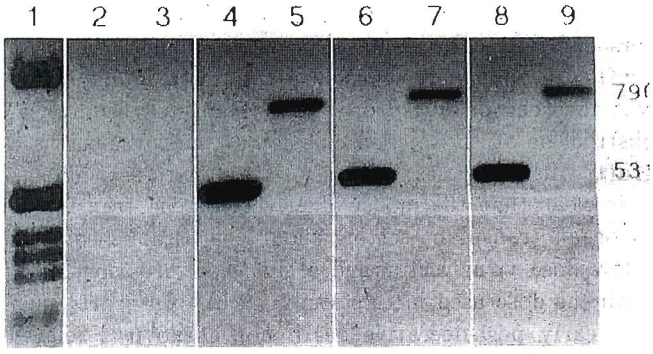

Fig. 2 In the samples obtained from group 2 animals, transfected gene sequence was examined by southern blot analysis using synthetic oligomers with sequences specific for hIL-7 DNA and the neoR genes. Proviral IL-7 DNA (531 kb) and neoR (790 kb) were found in the packaging cell line (positive controls, lane 4 to 5) and in reduced-size liver grafts perfused transfected with JZEN hIL-7/tk neo (lanes 6-9) at POD7. Whole-size graft perfused with the same vector did not demonstrate these sequences (lanes 2 and 3). The DNA ladder marker was run in lane 1.

X-gal staining

In group 1 animals, positive staining was detected in the hepatocyte until POD 14. The ratio of positively stained hepatocyte peaked at POD 2 (30 to 50% of total hepatocytes) and decreased thereafter. No staining was observed after POD 14 (Fig. 3).

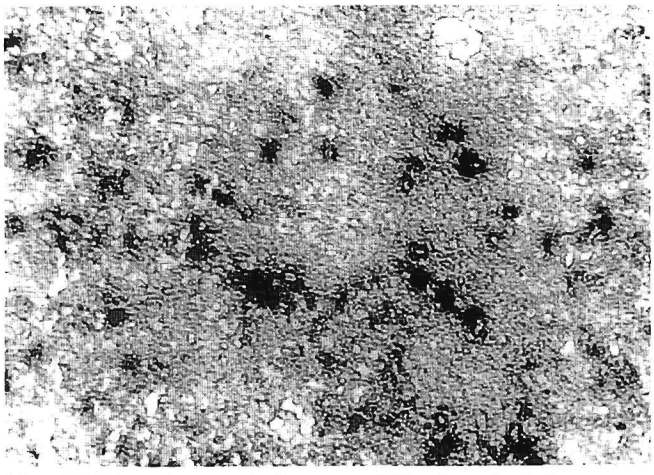

Fig. 3 Light microscopic findings of X-gal stained frozen sections obtained from the implanted liver graft (POD2) perfused ex-vivo with $1x10^{10}$ pfu of AdHCMVsp1LacZ. The cells expressing β-galactos-idase turned blue in the presence of X-gal

DISCUSSION

Although the retro-viral vector has been extensively used in gene transfer to the hematopoietic cells[3], gene transfer to the solid organ was rarely reported[8]. Since retrovirus-mediated gene transfection requires target cell replication at the time of transfection, solid organs might be the last candidate for a gene transfection with retro-viral vectors. However, the liver has unique capability of rapidly regenerating after the partial resection. Thus in this report, we have used the reduced-size liver graft, in addition to the whole-size liver graft, to obtain a post transplant graft regeneration for successful gene trasfection. As was expected, retro-viral mediated gene transfection was possible only in the reduced-size graft, but not in the whole-size liver graft. On the contrary, adeno-viral vector did not require the reduced-size liver graft for a sccessful gene transfection[4]. In our previous report, terminally differentiated cardiac muscle could also be transfected with the adeno viral vector[5] in the model of heterotopic heart transplantation. Thus in solid organ model of gene transfer, the adenovirus vector might be useful for acquiring successful gene transfection.

Time dependent and dose dependent increases in gene transfection have been reported both with retro- and adeno- viral vectors[5, 10]. To fulfill these conditions in a transplant setting, we have used the ex-vivo perfusion of the liver graft, during cold ischemia time. Although we did not use a preservation solution, such as University of Wisconsin solution (UW solution), feasibility of gene transfection has been also reported in this solution[9, 10]. Thus the ex-vivo perfusion with UW solution might contribute to the effective transfection of the desired gene sequences to the graft organ.

No specific inflammatory finding which might be attributed to the adeno- or retro- viral infection was observed in the histology of the liver grafts. However, increases in both the dose of virus-vector and preservation time were found to be contributory factors to a poor graft function after reperfusion. Based on our experience in a rat liver transplant model, no liver graft

perfused with more than $2x10^{10}$ pfu of adeno-viral vectors survived after 7 days post-transplant. In these grafts, the initial reperfusion after graft implantation was macroscopically poor, which suggested a physical obstruction of the microcirculation of the virus-perfused grafts with the virus vectors. Based on these findings, it is clear that the model of effective gene transfer in an organ transplant setting should be established with a low dose virus-vector and a short preservation time. With the dose used in this study ($1x10^{10}$ pfu/graft), neither early post-operative death nor adenovirus related systemic infection was observed in any of the recipients.

In conclusion, adeno- and retro-virus vectors successfully transmitted its cDNA into the liver graft by the ex-vivo perfusion of the liver grafts. However, the retrovirus vector required a graft reduction for achieving successful gene transfection. In the future, this methodology may allow for graft modulation in the early post transplant period.

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