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Adenovirus-mediated gene transfer using in-situ perfusion of the liver graft

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Abstract To establish an efficient technique for adenovirus-mediated gene transfer in liver transplantation, we evaluated the in situ perfusion of liver grafts. The grafts were perfused in situ with 1×10^{10} of E1-deleted, replication-defective adenoviral vectors encoding the LacZ gene driven by the human CMV promoter, either through the hepatic artery (group 1) or the portal vein (group 2). Group 3 animals served as negative controls; their liver grafts were perfused with lactated Ringer's solution through the portal vein. PCR confirmed the presence of viral DNA in every graft perfused with viral vectors. In X-gal staining, positive staining was observed almost exclusively at the portal triad in group 1, whereas in group 2 minimal staining was observed, predominantly in the parenchymal area. Protein production from the transfected gene was confirmed by a functional protein assay; the values were 0.16 % ± 0.07 % liver protein in group 1, 0.13 % ± 0.02 % in group 2, and 0.007 % ± 0.0003 % in group 3 on postoperative day 2. In conclusion, in situ perfusion of the viral vectors through the hepatic artery resulted in an effective expression of the transfected gene, predominantly at the portal triad.

Key words Gene transfer, adenovirus, liver transplantation · Adenovirus, gene transfer, liver transplantation · Liver transplantation, adenovirus, gene transfer

Introduction

Genetic modification of a grafted organ may provide an alternative technique for mitigating rejection or damage to the graft in allogeneic or xenogeneic transplantation by introducing gene expression of immunosuppressive cytokines or radical scavengers. Recently, replication-deficient adenoviruses have been increasingly used as a shuttle vector for gene transfer because of their ability to infect a wide range of target cells, regardless of the cell cycle, and because of their high viral stock titers [7].

Adenovirus-mediated gene transfer can be effectively achieved in the liver by increasing the duration and/or dose of viral exposure [4, 6, 11]. However, these strategies may also increase damage to the graft [6] and thus result in poor graft function. In order to establish an efficient gene transfer in the transplant setting, virus-mediated gene transfection should be performed with both a decreased duration and a decreased dose of viral exposure.

Ex vivo perfusion of grafts for virus-mediated gene transfection was tested in our previous studies [12, 13]. As much as 50 % of the ischemia time was spent harvesting the graft before starting the virus-mediated gene transfection [12, 13]. Since in situ perfusion is performed at the beginning of cold ischemia, the perfused graft can utilize the entire ischemia time for gene transfection, which might conversely result in a shortening of the cold ischemia time for gene transfection.

A unique feature of the liver is that it has a blood supply from both the portal vein and hepatic artery, and these two vessels are different in their anatomical distri-

butions in the liver [10]. We would thus expect the viral vectors perfused through either of these vessels to be delivered to different areas and cell populations in the liver. The aim of this study was to test the efficacy of in situ perfusion of the liver graft with a viral vector, either through the portal vein or the hepatic artery, while harvesting the liver from the donor.

Materials and methods

Animal model

Adult Wistar rats, 10–16 weeks old, were purchased from Ryukyu-Biotec (Okinawa, Japan). All animals used in our studies were handled humanely, in accordance with animal experimental protocols approved by the Animal Care and Use Committee of the University of Ryukyu. The animals were housed in microisolator cages in a virus-free facility and were fed laboratory chow.

Harvesting of the liver grafts and in situ perfusion

Prior to the surgical procedure, all donor rats were injected with 1 ml of lactated Ringer's (LR) solution containing 100 units of heparin. The abdominal cavity was entered through a midline incision, and the abdominal aorta (AO) was isolated and cannulated with a polyethylene catheter. The superior mesenteric artery (SMA) and bilateral renal artery (RA) were isolated, depending on the experimental protocol. Next, the supraceliac abdominal aorta was clamped, followed by a dissection of the diaphragma and intrathoracic inferior vena cava. Five milliliters of LR containing 100 units of heparin was then perfused through the abdominal aorta (rapid flush technique [14]). The grafts were further perfused in situ with the viral vector, either through the portal vein (PV) or hepatic artery (HA), depending on the experimental protocol. The liver grafts were harvested after gene transfection following routine methods. The total cold ischemia time of the grafts was adjusted to 1 h after the initial perfusion of the grafts.

Orthotopic liver transplantation

Orthotopic rat liver transplantation was performed using previously described techniques [9] under sterile conditions and under light ether anesthesia. Each recipient rat was isolated in a private cage and administered tobramycin, 1 mg/day, subcutaneously for 2 consecutive days post transplantation.

Viral vectors

The adenoviral vector AdHCMVsp1LacZ contained an expression cassette of the *E. coli* lacZ, under control of a CMV promoter and upstream of a polyadenylation signal. The expression cassette replaced the entire viral E1A region (early viral proteins) and most of the E1B region, thus rendering this virus replication defective. The virus was able to replicate in only 293 kidney embryonal cells that constitutively expressed the E1 proteins. The 293 cells were cultured in medium using the spindle cell culture technique, and the cell count was maintained at less than 4×10^5/ml by daily addition of the medium. Five liters of medium were thus obtained within 10 days. To generate high titer viral stocks, the 293 cells were first infected at a 5:1 pf u:293 cells with a spindle cell culture for 2 h. After a further infection of the 293 cells in 5 l of medium for 48 h, the cells were harvested in 50 ml of medium. The viral vector was recovered using three cycles of freeze-thawing. The viral stocks of 10^{10} pfu/ml were stored at –80 °C until use [5].

Experimental design

In groups 1 and 2, the liver grafts were perfused with 1×10^{10} pfu of AdHCMVsp1LacZ in 2 ml of LR at a rate of 1 ml/min. The animals transfected with the viral vectors were divided into two groups, depending on the viral perfusion procedure used. In group 1 rats, the SMA, bilateral RA, and proximal PV were clamped after rapid perfusion of the liver, and the viral vectors were injected through the AO, which was designed to be the isolated route for the HA. In group 2 rats, a polyethylene tube (PE-10, Imamura, Japan) was cannulated into the PV after rapid perfusion of the liver, and the viral vector was injected into the PV with a simultaneous clamping of the distal PV. Group 3 animals served as negative controls; their grafts were perfused in the same fashion as those in group 2, but without the viral vector.

The perfused grafts were orthotopically implanted into syngeneic recipients, and groups of five recipients were sacrificed on postoperative days (PODs) 2, 7, and 14 in groups 1–3. Tissue samples from the grafts were obtained from three different locations including the median lobe, left lateral lobe, and caudate lobe. In addition, serum and kidney samples were 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 and fixed with 1.25 % glutaraldehyde at 4 °C for 10 min. After the glutaraldehyde was removed, the preparations were immersed in 5-bromo-4-chloro-indolyl-β-D galactopyranoside (X-gal) solution and counterstained with eosin. The cells expressing β-galactosidase (β-gal) turned blue in the presence of X-gal, while the nonexpressing 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 ($\times$ 400) in the liver.

Polymerase chain reaction (PCR) analyses

Genomic DNA was obtained from homogenized frozen sections using phenol-chloroform extraction, suspended in TRIS-EDTA buffer, and then stored at –70 °C. The positive control consisted of DNA extracted from AdHCMVsp1LacZ-producing 293 cells. One microgram of DNA was amplified by PCR in a total volume of 50 ml that 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, Norwalk, Conn., USA). PCR was performed in a DNA thermal cycler (Perkin-Elmer). The amplification profile consisted of 30 cycles of denaturing for 1 min, the annealing of primers at 60 °C for 2 min, and extension at 72 °C 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. The PCR products obtained in this manner were then analyzed by gel electrophoresis.

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

To quantify the production of recombinant β-gal, an enzymatic assay was performed on liver protein. An aliquot of the total liver protein was incubated in ONPG buffer (0.1 M $MgCl_2$, 4.5 M b-mercaptoethanol, 4 mg/ml o-nitrophenyl-b-D-galactopyranoside, 0.1 M Na_2PO_4) at 37 °C for 30 min. The reaction was then halted by the addition of 1 M 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-gal. The results were then expressed as a percent β-gal per total cellular protein.

Results

Graft survival and light microscopic findings

Postoperative death related to technical failure occurred in less than 5 % of all recipients; they were excluded from the study analysis. The light microscopic study did not reveal any difference between the three groups. No specific inflammatory findings, which might be attributed to the adenoviral infection, were observed in any of the grafts (data not shown).

DNA PCR

Using PCR, DNA extracted from the liver graft or the kidney was evaluated for the presence of LacZ sequences. All liver grafts in groups 1 and 2 demonstrated LacZ sequences throughout the experiment, as did the positive adenovirus-producing 293 cells. A band corresponding to the sequence between the LacZ oligoprimers was seen at 1036 bp (Fig. 1). All grafts in groups 1 and 2 demonstrated LacZ sequences, while none in group 3 did. One-fifth of the rats in groups 1 and 2 showed LacZ sequences in the recipient's kidney; the other rats showed no detectable band in the kidney.

ONPG Assay

The quantity of virus-derived, recombinant β-gal protein in each group was determined by comparing its optical density with the optical density of purified *E. coli* β-gal using ONPG assays (Fig. 2). The levels of recombinant β-gal protein were 0.16 % ± 0.07 % liver protein in group 1, 0.14 % ± 0.02 % in group 2, and 0.007 % ± 0.003 % in group 3 on POD 2. The levels in groups 1 and 2 subsequently decreased; they were 0.05 % ± 0.009 % and 0.06 % ± 0.03 % in groups 1 and 2, respectively, on POD 7. On POD 14, the recombinant β-gal protein levels further decreased and were identical with those in group 3 (0.008 % ± 0.006 % liver protein in group 1 and 0.008 % ± 0.005 % in group 2). In group 3 (negative control), the grafts showed stable, low levels that did not exceed 0.01 % liver protein throughout the experiment.

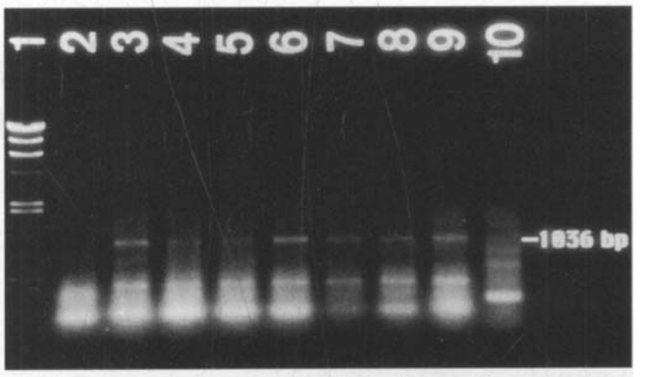

Fig. 1 In the samples obtained from group 1,2, and 3 rats, the transfected gene sequence was examined by PCR using synthetic oligomers with sequences specific for *E. coli* β-gal. The inserted β-gal gene (1036 bp) was detected in the viral packaging cell line (*lane 3*), and in the vector-perfused liver grafts of groups 1 and 2 (*lane 4* group 1 on POD 2, *lane 5* group 1 on POD 7, *lane 6* group 1 on POD 14, *lane 7* group 2 on POD 2, *lane 8* group 2 on POD 7, *lane 9* group 2 on POD 14), but not in the grafts of group 3 (*lane 2*). The DNA ladder marker was run in lane 1

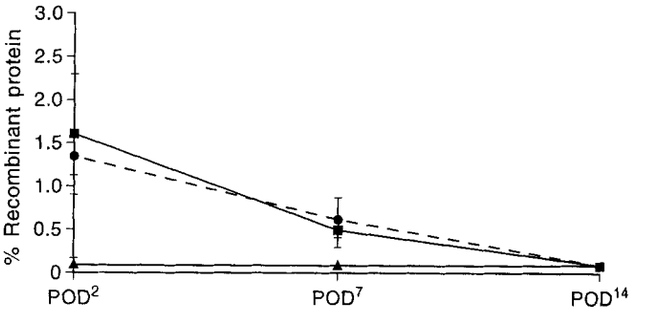

Fig. 2 Recombinant protein (β-gal) production in groups 1–3, represented as a percentage of total extracted liver protein. —■— Group I; —●— group 2; —▲— group 3

X-gal Staining

In group 1 rats, X-gal staining showed positive staining almost exclusively in the periportal area, bridging the adjacent portal areas (Fig. 3A, B). At the same time, group 2 rats showed a limited number of positively stained hepatocytes; these were predominantly observed in the parenchymal area (zones 1–2), without any positive staining in the periportal area (Fig. 3C). In addition, group 3 rats showed no staining in any of the samples (0 %) throughout the experiment.

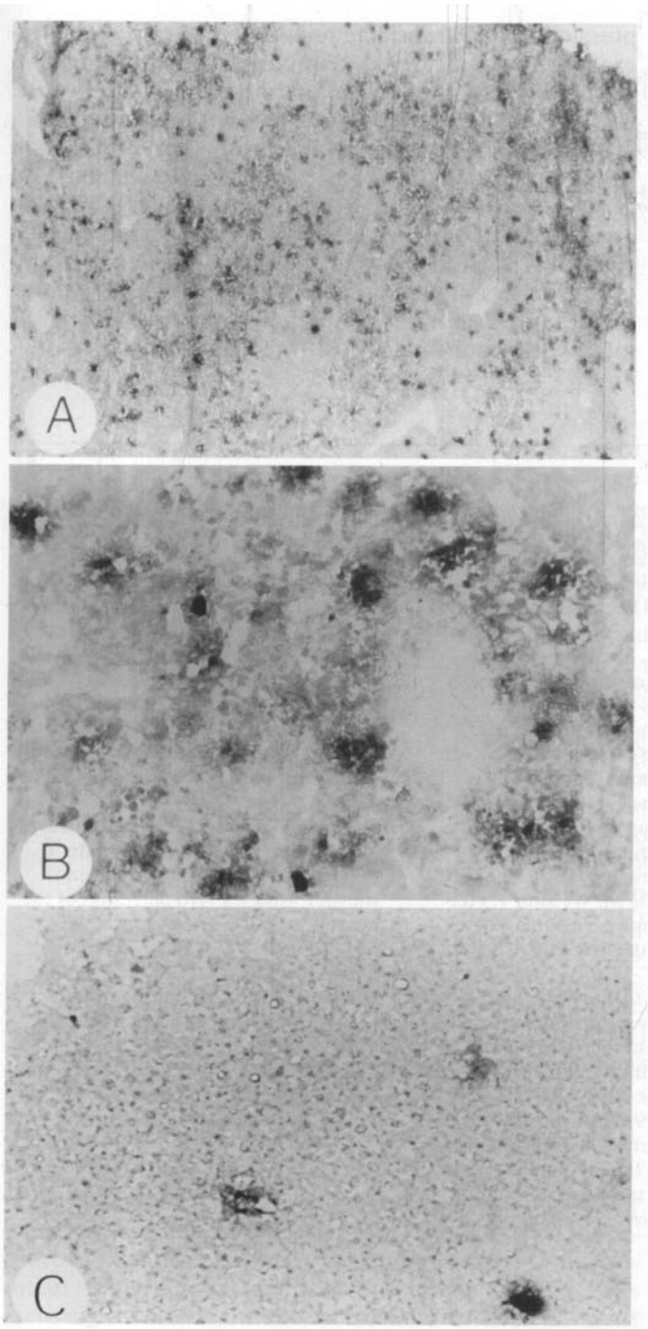

Fig. 3A–C Light microscopic findings of X-gal-stained frozen sections obtained from the implanted liver graft. The cells expressing β-galactosidase turned blue in the presence of X-gal: **A** group 1, POD 2 ($\times 100$); **B** group 1, POD 2 ($\times 400$); **C** group 2, POD 2 ($\times 100$)

Discussion

Gene transfer to a grafted organ prior to implantation may be used to genetically modulate allogeneic or xenogeneic grafts in the transplant setting. In adenovirus mediated gene transfection, both time-dependent and dose-dependent increases in gene expression have been reported [4, 6, 11]. However, increases in the dose of viral vector and in the preservation time were found to contribute to poor graft function after reperfusion. Based on our experience with a rat liver transplant model, no liver graft perfused with more than 2×10^{10} pfu of vectors has survived for more than 7 days post-transplantation. In these grafts, the initial reperfusion after graft implantation was macroscopically poor, thus suggesting a physical obstruction of the microcirculation of the virus-perfused grafts with the viral vectors. Based on these findings, it is clear that a model of effective gene transfer in an organ transplant setting should be established with a low-dose viral vector and a short preservation time. With the dose used in this study (1×10^{10} pfu/graft), neither early post-operative death nor adenovirus related systemic infection was observed in any of the recipients.

Another strategy for improving gene expression in a grafted organ has been to extend the duration of viral exposure, which also means a longer preservation time for the graft. With ex vivo perfusion of the graft, at least 30 min was required before viral transfection could be performed in a rat liver transplant model [12]. However, with in situ perfusion of the graft, viral transfection is performed at the initiation of cold ischemia, and, thus, the entire ischemic time can be used for the viral transfection. Moreover, rapid in vivo cooling with the in situ perfusion technique prior to harvesting tends to decrease damage to the graft [15]. During the initial 30 min of cold ischemia gained with the in situ perfusion technique, all of the harvesting procedures can be performed with a simultaneous gene transfer.

Hepatic ATP levels and energy charge values have been reported to rapidly decline after initiation of warm or cold ischemia [3]. Since the infection process of the adenovirus requires host ATP [2, 8], a rapid decline in hepatic ATP during cold ischemia may affect the adenoviral vector's ability to infect cells. Although we do not have data directly showing the effect of hepatic ATP on adenovirus-mediated gene transfer, the same dose of viral vectors showed improved performance with in situ perfusion compared to ex vivo perfusion in X-gal staining (data not shown).

The liver has a dual blood supply – from the portal vein and from the hepatic artery – and these vessels are different in their distributions in the liver. In this experiment, we perfused the grafts with the same dose of viral vector, whether it went through the hepatic artery (group 1) or the portal vein (group 2). Since the hepatic

arteriole forms the capillaries surrounding the bile ductule which, in turn, constitutes the peribiliary plexus [10], the perfused adenoviral vector is also delivered to the peribiliary plexus. Since the biliary system is an important target of hepatic allograft rejection [1], local delivery of the desired genes (i.e., immunosuppressive cytokines) to the biliary system could be used therapeutically to suppress acute rejection. In the present experiment, the distribution of the expressed protein from the transfected gene was evaluated with X-gal staining. Although we could not specify or quantitate the difference in the amount of expressed protein among the different cellular populations in the liver, the exclusive expression of the transfected gene on the portal triad in group 1 may indicate that viral transfection through the hepatic artery is effective in transfecting the desired gene to the portal triads. Since the portal triads contain a variety of nonparenchymal cells, viral transfection through the hepatic artery would thus contribute to gene transfection to nonparenchymal cells in the liver.

In conclusion, the adenoviral vector successfully transmitted its cDNA into the liver graft using in situ perfusion techniques, especially through the hepatic artery. In the future, this methodology may allow for graft modulation in the early post-transplant period.

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