ADENOVIRUS-MEDIATED GENE TRANSFER TO LIVER GRAFTS

AN IMPROVED METHOD TO MAXIMIZE INFECTIVITY

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Background. Adenoviral gene therapy in liver transplantation has many potential applications, but current vector delivery methods to grafts lack efficiency and require high titers. In this study, we attempted to improve gene delivery efficacy using three different delivery methods to liver grafts with adenoviral vector encoding the LacZ marker gene (AdLacZ).

Methods. AdLacZ was delivered to cold preserved rat liver grafts by: (1) continuous perfusion via the portal vein (portal perfusion), (2) continuous perfusion via both the portal vein and hepatic artery (dual perfusion), and (3) trapping viral perfusate in the liver vasculature by clamping outflow (clamp technique).

Results. Using 1×10⁹ plaque-forming units of AdLacZ (multiplicity of infection of 0.4), transduction rate in 3-hr preserved liver grafts, determined by 5-bromo-4-chromo-3-indoly3-D-galactopyranoside staining and β-galactosidase assay 48 hr after transplantation, was best with clamp technique (21.5±2.7%, 66.6±19.4 U/g) and dual perfusion (18.5±1.8%, 66.6±19.4 U/g) and portal perfusion (18.5±1.8%, 66.6±19.4 U/g). Further studies using clamp technique demonstrated a near-maximal gene transfer rate of 30% at multiplicity of infection of 0.4 with prolonged cold ischemia to 18 hr. Transgene expression was stable for 2 weeks and slowly declined to 7.8±12.1% at day 28. Lack of inflammatory response was confirmed by histopathological examination and liver enzymes. Transduction was selectively induced in hepatocytes with nearly no extrahepatic transgene expression in the lung and spleen.

Conclusions. The clamp technique provides a highly efficient viral gene delivery method to cold preserved liver grafts. This method offers maximal infectivity of adenoviral vector with minimal technical manipulation.

The introduction of functional genes into organ grafts before transplantation has been an attractive approach to modulate deleterious posttransplant events. The range of potential therapeutic applications includes amelioration or prevention of host versus graft immune reactions and reduction of graft ischemic and preservation injury. Effective gene therapy requires a reliable method of gene transfer that efficiently inserts target genes to the parenchymal and/or nonparenchymal cells in grafts to produce required levels of proteins. Viral vectors, especially replication-defective adenoviruses, have been favored because of their ability to readily infect nonproliferating cells. In contrast, retroviral vectors infect only proliferating cells. Although adenoviral-mediated recombinant gene expression has been limited to a short period after gene transfer, this approach may provide advantages in modifying the critical early posttransplant period when intense immunological events and ischemic graft injury occur.

Previous studies using adenoviral vectors have demonstrated in vivo infectivity in experimental liver transplant settings by perfusing grafts with viral vectors (1–3). However, these ex vivo or in situ perfusion techniques required high viral titers (1×10⁹ plaque-forming units [pfu]) to induce effective viral infection, most likely as a result of the limited contact of viral particles with the graft vasculature and the resulting escape of the majority of viral vector from the graft. Prolonged incubation with viral vector has been shown to induce higher levels of viral infection to the liver, but efficiency remained low (~50% infection at virus titer 5×10⁹) (2). In addition, previous methods of ex vivo perfusion have included a complicated circuit, which may not be suitable for clinical application, and increases the chance of mechanical graft injuries.

In this study, we developed an improved gene delivery method in the liver transplant setting that allows near-maximal infectivity of adenoviral vector by trapping vector perfusate within the liver graft for the duration of the cold preservation period. We compared this new technique with two other gene delivery methods consisting of continuous viral vector perfusion via the portal vein alone or dual perfusion via the portal vein and hepatic artery. Several variables affecting transduction efficiency were analyzed: (1) effects of different viral titers, (2) incubation times, and (3) delivery techniques. After transduction, we examined peak duration of gene expression, cell specificity, as well as the extent of damage to the liver as a result of viral infection.

MATERIALS AND METHODS

Viral Vector

The E1- and E3-deleted adenovirus, AdLacZ*, contains the β-galactosidase gene under a cytomegalovirus (CMV) promoter (GenVec, * Abbreviations: AdLacZ, adenoviral vector encoding LacZ; CMV, cytomegalovirus; LR, lactated Ringer's, MOI, multiplicity of infection; pfu, plaque-forming unit(s); SGOT, serum aspartate aminotransferase; SOPT, serum alanine aminotransferase; UW, University of Wisconsin; X-gal, 5-bromo-4-chromo-3-indoly3-D-galactopyranoside.)

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incubation time would improve viral infectivity, liver grafts in group 3 were incubated for a total cold ischemia time of 3 or 18 hr at 4°C with either 2 × 10^9 or 1 × 10^9 pfu of AdLacZ before transplantation (n = 3 for each incubation time). Animals were killed 48 hr after transplant.

**DURATION OF GENE EXPRESSION.** Liver grafts in group 3 were perfused with 1 × 10^9 pfu of AdLacZ (MOI 0.4) and incubated for 18 hr at 4°C. After transplantation, animals were killed at 1, 2, 7, 14, and 28 days (n = 3 for each time point) to examine the duration of transduced gene expression in the liver. Control livers were perfused with UW solution without viral vector and samples were obtained 48 hr and 14 days after transplantation. Blood samples (0.7 ml) were collected from the tail vein 6 and 12 hr postoperatively, and then on postoperative days 1, 2, 5, 7, and 14 (n = 4 each time point for experimental animals; n = 3 each time point for controls).

**SAMPLING.** At death, each liver graft was flushed with LR solution before sampling. Left, middle, and right liver lobes, spleen, and lung were fixed in formalin for routine histopathology, fixed in 2% paraformaldehyde for immunohistochemical staining, or snap-frozen in liquid nitrogen for recombinant β-galactosidase protein analysis.

**5-Bromo-4-chromo-3-indolyl-β-D-galactopyranoside (X-Gal) Staining**

Liver samples fixed in 2% paraformaldehyde for 2 hr at 4°C were transferred to 30% sucrose and kept overnight at 4°C. Tissues were snap-frozen in OCT using a bath of isopentane immersed in liquid nitrogen, cut on a cryostat to 7 μm thickness, and mounted on glass slides. X-Gal (Boehringer-Mannheim Biochemical, Indianapolis, IN) solution was applied to tissue sections, and slides were incubated in a humidified chamber at 37°C overnight. Cells expressing β-galactosidase turned blue in the presence of X-gal. Tissue nuclei were stained with propidium iodide for 30 sec at room temperature. The percentage of cells positive for X-gal staining was assessed by counting the number of positively staining cells and the total number of nuclei at 20× magnification. At rates of infection >10%, the density of X-gal was too great to count individual cells. Therefore, infection rates in these samples were assessed at 20× magnification by measuring the total cross-sectional area and the area occupied by positively staining cells with the assistance of a computer quantification program (Optimas; Optimas Co., Seattle, WA). Eighteen to 30 fields were assessed for each liver, with equal number of fields for each lobe (right, middle, and left).

**Histopathology**

Formalin-fixed liver samples were embedded in paraffin and cut into 4-μm-thick sections. Tissues were stained with hematoxylin and eosin, and slides were assessed for inflammation and tissue damage.
Liver Function Tests

To assess hepatic function and cellular injury after gene transfer and preservation, serum aspartate aminotransferase (SGOT) and serum alanine aminotransferase (SGPT) were measured using the Opera clinical chemistry system (Bayer Co., Tarrytown, NY).

Recombinant Protein Detection

β-Galactosidase activity was determined using a β-galactosidase enzyme assay kit (Promega, Madison, WI). Briefly, samples from the middle lobe of livers were homogenized and total protein was extracted by incubation with a reporter lysis buffer. Liver protein was incubated with o-nitrophenoxy-β-D-galactopyranoside at 37°C for 30 min. Optical density of the solution was read at 420 nm, and compared to a standard curve generated from known amounts of recombinant β-galactosidase. Liver graft β-galactosidase activity was recorded as units (U) of β-galactosidase activity per gram (g) of total cellular protein.

Analysis of Cells Expressing Transferred Gene

A portion of liver sample infected with AdLacZ was separated into hepatocyte and nonparenchymal cell fractions according to the method previously described (8). Briefly, liver tissue was digested with L-15 medium containing 0.1% collagenase (Boehringer-Mannheim) and 0.005% trypsin inhibitor (Sigma Chemical, St. Louis, MO) at 37°C. Cell suspension was centrifuged at 400 rpm for 1 min and the pellet collected to isolate hepatocytes. Supernatant was centrifuged at 1200 rpm for 8 min to isolate nonparenchymal cells. After a brief red blood cell lysis step, debris from the nonparenchymal cell fraction was removed by centrifugation at 2000 rpm for 30 min using a 30% Percoll (Sigma) gradient. Hepatocyte and nonparenchymal cell fractions were fixed with 2% paraformaldehyde for 10 min and resuspended in X-gal staining solution. Cells were incubated at 37°C overnight. Cytospins were made, and cells analyzed for X-gal positivity.

Statistical Analysis

Results of infection rates and β-galactosidase activity are expressed as the mean ± standard deviation of the mean. Group comparisons were performed using ANOVA (analysis of variance) test. Differences were considered significant at P<0.05.

RESULTS

Effect of delivery methods on infection rate. The evaluation of different methods of gene delivery using a viral titer of 1×10⁹ pfu revealed substantial differences in viral infectivity between groups 1, 2, and 3 by X-gal staining (Fig. 1). As shown in Figure 1A, control livers did not stain positive for X-gal. Portal perfusion alone (group 1) gave a transduction rate of 8.8±2.5% (Fig. 1B). Dual perfusion of the portal vein and hepatic artery (group 2) yielded a 18.5±1.8% transduction rate (Fig. 1C), whereas the clamp technique (group 3) provided the greatest delivery at 21.5±2.7% (Fig. 1D). The number of LacZ gene-transferred cells was significantly increased in group 2 (P<0.005) and group 3 (P<0.001) animals, compared to that in group 1. Gene transfer was predominantly seen in liver cells near the portal triads in groups 1 and 2 animals, whereas it was more dispersed through the entire liver lobes in group 3 animals. Results obtained from the β-galactosidase enzyme assay (Fig. 2) independently confirmed X-gal staining data. Group 1 animals demonstrated an enzyme activity of 19.7±15.4 U/g, whereas activity increased 3-fold to 66.6±19.4 U/g (P=0.002 vs. group 1) in group 2, and was highest at 81.1±3.6 U/g in group 3 (P=0.008 vs. group 1, NS vs. group 2). Although similar transduction rates were seen with the dual perfusion and clamp techniques, less technical manipulation was required in the clamp technique of gene delivery. Accordingly, the clamp technique was then used in further experiments to study other variables affecting gene transduction.

Effect of viral titer on gene delivery. Similar to the previous report by Shaked et al. (2) using the portal infusion technique, a clear positive correlation between viral titer and transduction rate of liver cells was observed with the clamp technique in group 3 animals. With infusion of 1×10⁸ pfu of AdLacZ (MOI=0.04), the gene transfer rate was 3.9±0.7%. By using higher viral titers of 2×10⁸ pfu (MOI=0.08) and 1×10⁹ pfu (MOI=0.4), the percentage of liver cells positive for LacZ increased to 6.2±1.7% and 21.5±2.7%, respectively.

Evaluation of incubation time. Inasmuch as the cold ischemia time of liver grafts in the clinical setting often varies
from 3 hr to 20 hr, we evaluated the effect of different incubation times (cold ischemia time) on gene delivery. Group 3 livers incubated with $2 \times 10^8$ pfu of viral vector for a total cold ischemia time of 3 or 18 hr demonstrated 6.2±1.7% and 5.2±1.3% infectivity, respectively (Fig. 3). When increasing the viral titer to $1 \times 10^9$, livers incubated for 3 hr showed 21.5±2.7% positive for LacZ, and prolonging cold ischemia time to 18 hr resulted in a significant increase in positive staining to 29.9±5.1% ($P<0.01$ vs. 3-hr incubation). As the theoretical maximum of infectivity at MOI 0.4 ($1 \times 10^9$ pfu) is 40%, these results demonstrate that near-maximal transduction is obtained by using this gene delivery method.

Duration of gene expression. To determine the duration of transgene expression in the liver grafts, we performed a posttransplant time-course study. When the liver was infected with $1 \times 10^9$ pfu of AdLacZ for the 18 hr incubation period, a constant gene transfer rate near 30% was maintained at 1, 2, 7, and 14 days after transplant (Fig. 4A). At day 28 after transplant, the percentage of positive staining decreased to 7.8±12.1%, with considerable variability between different animals. Recombinant protein analysis confirmed a stable level of $\beta$-galactosidase activity at roughly 100 U/g through day 14 (Fig. 4B). This value decreased to 40.2±49.4 U/g by day 28 after transplant.

Liver injury. As adenovirus has been reported to induce an inflammatory reaction in situ (9), we examined the effect of virus delivery on liver enzymes and histopathology. Serum SGOT and SGPT levels peaked 12–24 hr after transplant in group 3 and control animals (Fig. 5, A and B). Liver enzymes returned to normal values by day 3 after transplant, and remained low through 14 days. These results demonstrate that no liver damage was induced by LacZ transduction. Histopathological examination of the liver grafts revealed spotty necrosis in both group 3 and control livers at 48 hr after transplant. At days 14 and 28, liver grafts demonstrated normal appearance. No inflammatory infiltrate was noted in either LacZ-infected or control animals throughout the course of the study.
Analysis of cells expressing transferred gene. To determine which liver cell types were being infected by the adenovirus, we isolated hepatocyte and nonparenchymal cell fractions from transfected livers. After isolation and X-gal staining of the two fractions, a clear difference in positivity was noted among cell types (Fig. 6, A and B). Positive cells were abundantly found in the hepatocyte fraction, whereas few positive cells (<1%) could be observed in the nonparenchymal cell fraction, suggesting that the AdLacZ selectively targets hepatocytes in the liver.

Escape of vector to extrahepatic organs. To determine if the targeted gene delivery remained organ-specific, we stained additional organs for LacZ expression. Few cells (~1 per section) in the lung and spleen were found to be positive for LacZ gene expression with X-gal staining (Fig. 6, C and D). This lack of expression in extrahepatic organs suggests that the viral vector specifically targets hepatocytes using our delivery method.

DISCUSSION
This study demonstrates a highly efficient in vivo gene transfer method, which allows maximal infectivity of adenoviral vector to liver grafts. The procedure is simple, does not require extra instrumentation, and is clinically applicable. In previous studies, gene delivery to 10-30% cells in rat liver was obtained only when very high titers of adenoviral vector (5×10^10 pfu) was delivered via portal perfusion (1, 2). These titers correspond to an MOI of 20, based on the estimated total number of rat liver cells as 2.5×10^9 (7), suggesting that the majority of infused vector fails to infect liver cells. Using the delivery method described here, similar infectivity is achieved with substantially lower titer of viral vector (1×10^9 pfu, 50-fold less). This infectivity, at an MOI of 0.4, was near a theoretical maximum transduction rate of 40%. The superiority of this delivery method compared to previous studies is based on the following: (1) vector infusion via both the portal vein and hepatic artery allows delivery of viral vector to areas of hepatic microcirculation that were inaccessible by single perfusion, likely as a result of vasoconstriction caused by cold preservation; (2) vector-target cell contact is enhanced by trapping of vectors in the liver microcirculation; and (3) longer incubation of vector in the graft microvasculature can be achieved for the cold preservation period to further enhance gene transduction. As the conventional perfusion method may be difficult to apply in a clinical setting, the clamp technique, which involves less technical difficulty and instrumental complexity, can readily be applied to a clinical transplant situation.

As has been demonstrated previously, and now shown in our study, a proportionally larger percentage of liver cells could be transfected by increasing the MOI (2). Using the method described here, transduction efficiency at each successive viral titer reaches near-maximal infectivity.

Although it has been widely used for gene therapy, a major disadvantage of the adenovirus vector has been the low level production of viral late proteins and the subsequent host immune responses against these protein antigens. These in-

Figure 6. X-Gal staining of hepatocyte (A) and nonparenchymal cell (B) fractions. A large number of hepatocytes stained positive with X-gal, whereas only a few were positive in the nonparenchymal cell fraction. Positive cells were scarcely found in the lung (C) or spleen (D). Figure shows 1×10^9 pfu of AdLacZ, 48 hr after transplant. Original magnification, ×20.
duce local inflammation, destruction of transduced cells, and formation of adenoviral vector-specific antibodies, resulting in the limited persistence of transgene expression, as well as the inability to repeat vector administration. These consequences were delineated in previous rodent studies involving liver transduction by adenoviral vectors infused into the hepatic or systemic circulation (9–11), and have led to the recent development of second generation adenovirus vectors, which contain additional deletions of early transcription units (12, 13). LacZ expression in our study started to decline 3–4 weeks after delivery. However, we did not observe hepatic inflammatory infiltrates or liver-related enzyme elevation, which have been shown to be associated with disappearance of transgene expression (13, 14).

The explanation for a lack of inflammatory response in this study is not entirely clear. Interestingly, Olthoff and co-workers (15) have recently observed similar findings in rat liver grafts after ex vivo perfusion of adenoviral vectors through the portal vein. This contrasts with the significant inflammatory response described after transduction of murine hepatocytes (10, 11), suggesting a species-specific response. These murine studies utilized in vivo systemic delivery of viral vector, with significant exposure of recipient lymphoid tissues to the virus. In contrast, transduction using the current ex vivo method was limited almost exclusively to hepatocytes, with minimal transgene expression in liver nonparenchymal cells and extrahepatic tissues, such as the lung and spleen. An immune response is based on a balance between potentially reactive lymphocytes and the composition, quantity, kinetics, and distribution of antigen within the host (16). Antigens that do not enter organized lymphoid tissues have been shown not to induce immune responses (immune indifference) (16), and viruses persisting in peripheral nonlymphoid tissues, such as hepatocytes in this study, may be ignored by the host immune system. Leakage of antigens into lymphoid tissues in murine studies may contribute to the activation of T cells to control infection (9).

The actual cause for the eventual loss of transgene expression despite the lack of a significant host inflammatory response against the virus is unknown. Others have shown that the CMV promoter becomes silent in a few weeks after gene transfer in a number of animal studies including immunocompetent and immunocompromised animals (17–19), retroviral (18, 20) and adenoviral (21) vectors, and different cell types (20, 21). Loser et al. (22) recently reported transcriptional inactivation of the CMV promoter in the mouse liver after adenoviral transduction, probably through the inactivation of the transcription factor NFκB. Whether this mechanism is occurring in the transplanted livers is uncertain, but it is at least possible that CMV promoter silencing may be in part responsible for loss of transgene expression in our experiment.

In summary, we have developed a method of gene delivery that allows near-maximal gene transfer efficiency to cold preserved liver grafts. Using the clamp technique, stable gene expression can be attained through 2 weeks after transplant, allowing future therapeutic genes to target the critical early posttransplant period. This technique allows the use of substantially lower particles of adenoviral vector than previously established methods of vector delivery, and is not associated with an inflammatory response to the virus. Studies are currently under way to determine the effectiveness of this technique of gene delivery using potentially therapeutic functional transgenes.

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