Efficient Adenoviral-mediated Ornithine Transcarbamylase Expression in Deficient Mouse and Human Hepatocytes

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Abstract

100% of primary human hepatocytes infected with an adenoviral vector carrying β-galactosidase expressed the exogenous gene. Expression was also achieved in > 40% of adult mouse hepatocytes in vivo.

Normal levels of activity were achieved in mouse ornithine transcarbamylase (OTC)-deficient primary hepatocytes using another adenoviral vector carrying human OTC cDNA. Study of OTC-deficient primary human hepatocytes from a single patient confirmed the utility of adenoviral delivery of OTC.

We describe adenoviral-mediated exogenous gene expression in human and mouse hepatocytes in vitro and in mouse liver in vivo. Data suggest that adenoviral vectors may be useful for correcting OTC deficiency. (J. Clin. Invest. 1993. 92:1580–1586.) Key words: ornithine transcarbamylase • human • liver • gene therapy • β-galactosidase

Introduction

Despite tremendous progress in medicine today, many genetic disorders remain without adequate treatment. A gene augmentation or replacement regimen is therefore an innovative and potentially new solution to this inadequacy.

We have previously proposed that the urea cycle defect ornithine transcarbamylase (OTC)1 deficiency is an excellent choice for gene therapy research, since it occurs frequently by new mutation, is cured by hepatic transplantation in some patients, has two mouse models available for experimentation, and the gene is well characterized (1, 2). Furthermore, drug therapy for the acute hyperammonemia that occurs during catatonic episodes has shown some benefit, suggesting that long- or short-term or partial OTC gene expression may have clinical utility if in vivo delivery and expression could be achieved.

In recent years, several reports have indicated the utility of defective adenoviral vectors for gene therapy (3–7). Adenoviruses have several features that make them attractive as potential gene therapy agents. They do not require cell proliferation to transfer gene expression (8), can presently accommodate ≤ 8 kb of foreign DNA, and can easily be produced and purified at titers > 109 ml (9). In addition, although adenovirus serotype 5 in subgenus C is a common cause of upper respiratory tract infection in children < 5 yr of age (10), this virus is not associated with serious illness in immunologically competent individuals or malignancies (8, 11–13). Furthermore, for more than two decades, unattenuated adenoviruses have been successfully used as oral vaccines (14). This evidence of the safety of adenovirus reinforces the potential significance of this system as a gene therapy tool. For human gene therapy purposes, evaluation of the vector of choice in human target tissue is essential. Until now, there have been no studies on the use of recombinant adenovirus in primary human hepatocytes despite several successful studies in rodents (3–7).

Methods

Construction of AdHCMVsp1LacZ and AdSRohOTC. The LacZ and the hOTC genes were inserted in the E1 region into different compensatory deletions. The deletions into which the genes were inserted were generated using the same initial plasmid, pXCI (15), which contains the first 5,788 bp of the adenovirus type 5 (Ad5) genome inserted into the BamHI and SalI sites of pBR322. pXC12, the plasmid into which the hOTC gene was inserted, was generated from pXCI by deleting Ad5 sequences from the PvuI site at 452 bp to the BglII site at 3328 bp, and inserting an XbaI cloning site into which SRohOTC was inserted (16). pHCMVsp1A, the plasmid into which the lacZ gene was inserted, was generated from pXCI by deleting Ad5 sequences from the XspI site at 339 bp to the AlwII site at 3,533 bp. This deletion removes the spII binding site (3,525–3,530 bp) from the protein IX promoter that was reintroduced 1 bp closer to the protein IX TATA box. Various unique cloning sites and the human cytomegalovirus (HCMV) promoter were introduced into the deletion. One other difference between pHCMVsp1A and pXCI is that ~ 400 bp of pBR322 sequences were removed between the EcoRI and BamHI sites. The lacZ gene with the SV40 poly A signal was inserted between the SalI and XbaI sites of pHCMVsp1A generating pHCMVsp1LacZ. Both pAdSRohOTC and pHCMVsp1LacZ were rescued into virus by cotransfection with pJM17 (17).

Hepatocyte cultures. Human liver segments preserved in University of Wisconsin solution (18) for liver transplantation were obtained after reduction through the liver tissue procurement and distribution system (19). Under aseptic conditions, ~ 40 g of the liver were removed, and hepatocytes were isolated according to Ledley et al. (20). Hepatocytes were infected with recombinant adenoviruses, AdHCMVsp1LacZ and AdSRohOTC, as described by Graham and
Prevec (9). Cells were exposed to the viral suspensions 48 h after plating for 1 h at room temperature with a range of multiplicities of infection, based on plaque-forming units (pfu) defined on the 293 cell line (21). Controls were subjected to the same infection conditions, except that virus-free suspension buffer was used (phosphate-buffered saline with Ca\(^{2+}\), Mg\(^{2+}\), and 10% glycerol). 1 h after infection, cells were washed with phosphate buffered saline followed by the addition of culture media. Mouse-derived hepatocytes cultures were prepared essentially as described by Grompe et al. (1).

**Histochemical staining.** Cells were fixed and evaluated for the expression of *Escherichia coli* β-gal enzyme activity after staining with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal stain), and α\(_1\) antitrypsin. Cultures were fixed in 0.5% glutaraldehyde (Sigma Immunochemicals, St. Louis, MO) and stained as described (22). For immunohistochemical staining, cultures were fixed in 0.5% glutaraldehyde and incubated with a 1:1,200 dilution of rabbit anti-α\(_1\) antitrypsin antibody (Atlantic Antibodies, Scarborough, ME). Immunoperoxidase staining was performed using the Vectastain ABC method (Vector Laboratories, Inc., Burlingame, CA), and hematoxylin was used as a counter stain.

**OTC assay.** Cells were harvested by scraping into 0.25 M Tris/HCl, pH 7.2, and total protein was extracted by repeated freeze thaw cycles.

*Figure 1. β-gal expression in vitro.* (C–G) Human hepatocyte cultures infected with AdHCMVsp1LacZ at 10, 20, 50, 100, and 200 moi, respectively. (A) Cultures stained immunohistochemically after incubation with α\(_1\) antitrypsin antibodies. (B) Control hepatocytes infected with AdSRahOTC at moi of 200.
OTC enzyme activity was measured according to Jones et al. (2) using 50 μg of protein for the assay in a final volume of 100 μl for 1 h.

In vivo viral delivery. 2-mo-old C3H/HENCRMTV mice were anesthetized and their abdomens were opened, exposing the portal vein or the spleen. Intraportal or intrasplenic injections with $1 \times 10^{10}$ and $2 \times 10^{10}$ pfu of AdHCMVsp1LacZ in 100 and 200 μl of viral suspension buffer (PBS++ plus 10% glycerol), respectively, or with virus-free suspension buffer were performed using a 30-gauge needle.

Frozen sections. Liver tissue was snap frozen by plunging into liquid nitrogen-cooled isopentane. 8-μm sections were fixed in 2.5% glutaraldehyde and stained with X-gal as described (22).

### Results

**Adenoviral-mediated gene expression in human hepatocytes.** Cultured human hepatocytes infected with recombinant adenovirus efficiently expressed exogenous genes. We constructed and used an Ad vector (AdHCMVsp1LacZ) carrying an early region 1 (E1) substitution of an expression cassette consisting of HCMV immediate early gene promoter driving bacterial βgal expression to infect human hepatocytes. Cultures were infected with AdHCMVsp1LacZ at different moi (Fig. 1, C-G). 100% of the cells expressed βgal when infected at an moi of 200 or 400 pfu/cell at 72 h and ≤2 wk after infection (Fig. 2). Cells infected at moi's of 10, 20, 50, and 100 yielded expression ranging from 2% to 84% (Fig. 2).

With respect to cell morphology (Fig. 1, B, C-G) and endogenous OTC activity (Fig. 3), infected cultures were comparable to controls. The culture conditions used select against non-hepatocyte liver cells (23), and > 95% of the surviving cells were hepatocytes, as shown by morphology and immunohistochemical staining with antibody to the hepatocyte-specific enzyme marker, α1 antitrypsin (Fig. 1 A).

**Adenoviral-mediated expression in vivo.** Adult mice were used to evaluate the efficiency of adenoviral gene transduction in vivo. Estimation of the proportion of cells expressing β-galactosidase (β-gal) from frozen sections was difficult because of the patchy distribution of expressing cells (Fig. 4 B). Some areas were densely stained, while others showed sparse staining with no apparent overall pattern. Therefore, for a more reliable estimate of transduction, livers were cultured, and 24 h after plating, they were fixed, stained, and evaluated (Fig. 4 A). Splenic (n = 8) or intraportal (n = 8) infusions of $1 \times 10^{10}$ and $2 \times 10^{10}$ pfu resulted in 21.6 ± 7.5 and 42.4 ± 6.7% of cells expressing βgal activity 72 h after infection, respectively. Livers perfused and stained in situ 72 h after infection showed an overall positive βgal staining with no confinements to any specific area or lobe of the liver (Fig. 5). No morphologic differences (gross or microscopic) were found between control (uninfected, AdSRahrOTC, or viral suspension buffer–mock infected) and AdHCMVsp1LacZ-infected mice, and no background staining was found in control livers (Fig. 1 B and Fig. 5).

**OTC correction in vitro.** Hepatocytes derived from sparse fur (spf) mice with > 80% reduction in endogenous OTC activity were infected 48 h after plating with AdSRahrOTC. 1 wk after infection, OTC activity was assayed. The percentage of OTC activity detected in hepatocytes infected with AdSRahrOTC ranging from 1 to 200 moi ranged between 26 and 151% relative to wild type–derived hepatocytes uninfected or infected with AdHCMVsp1LacZ (Fig. 6). AdSRahrOTC was also used to infect OTC-deficient primary human hepatocytes isolated from a severely affected patient undergoing liver transplant. Preliminary data show an increase in OTC activity 4 d after infection (Fig. 7).

**Discussion**

Our results show that essentially all of the primary human hepatocytes in culture that were infected with AdHCMVsp1LacZ at moi's of 200 or 400 expressed βgal activity. Ad mutants defective in postinfection functions, and to a much lesser extent, mutants deleted in E1, can show multiplicity-dependent leakiness in certain cells, particularly at very high pfu/cell ratio (24, 25). Exposure of human hepatocytes to the high moi's used in these studies did not alter endogenous OTC enzyme activity levels. Furthermore, no cytopathic effects were observed for as long as 2 wk after infection, and no morphological differences were observed between recombinant virus–infected and uninfected cultures, suggesting that significant levels of virus replication were not occurring.

In vivo, single injections of AdHCMVsp1LacZ (2 × $10^{10}$ pfu/ml) targeted to the liver in adult mice resulted in > 40% of
Figure 4. Adenoviral-mediated β-gal expression post in vivo portal vein infusion. Livers were analyzed 72 h after infection for β-gal expression. (A) Hepatocytes were cultured 72 h after infection and evaluated for β-gal expression. (B) Liver frozen section evaluated 72 h after injection.
cells expressing βgal in the liver. The percentage of cells expressing the inserted gene at 72 h after treatment exceeded levels recently reported by Jaffe et al. (62% in vitro and <2% in vivo) (5). This difference may relate to several factors, including the difference in vectors used, promoters, and the amount of recombinant virus injected in relation to the size of the animal (25–30 g/mouse vs 250–300 g/rat). We anticipate that with our vectors, even higher levels of activity may be obtained with minor technical modifications, such as increasing the moi, the time, or repeated inoculation.

Sparse fur mouse-derived primary hepatocytes, in which OTC activity is ~20% that of wild type, were fully corrected after infection with recombinant adenovirus carrying the human OTC cDNA under the control of the SRα promoter (26), and the levels of activity equaled or exceeded those of wild type controls. We report the first successful attempt for OTC gene augmentation in human hepatocytes derived from an OTC-deficient patient resulting in an increase in enzyme activity. The wild type levels of OTC expression achieved in this report are the first demonstration of complete correction for the deficiency in vitro. This is significant in terms of OTC gene therapy and in vivo targeting of the liver. We therefore predict, based

Figure 5. Whole liver was perfused in situ with PBS followed by 2.5% gluteraldehyde, PBS, and stain. After dissection, livers were incubated for an additional 2 h in X-gal stain. (Left to right) In vivo portal transfer of $2 \times 10^{10}$, $1 \times 10^{10}$ pfu of AdHCMVsp1LacZ, and $2 \times 10^{10}$ pfu AdSRαhOTC followed 72 h after infection, by in situ fixing and X-gal staining of the liver.

Figure 6. Livers of 2-mo-old spf (OTC-deficient) and C3H/HENCRMTV mice (wild) (Jackson Laboratories, Bar Harbor, ME) were used for the hepatocyte cultures. OTC-deficient hepatocytes were infected 48 h after plating at different moi's with AdSRαhOTC, AdHCMVsp1LacZ, or suspension media (mock) and evaluated for OTC enzyme activity 1 wk after infection ($n = 3$). Vertical error bars show standard deviations from the mean.

Figure 7. This is an autoradiograph of a TLC plate used to separate the product citrulline (arrow) from the substrate ornithine of the OTC enzyme reaction assay. Human hepatocytes were isolated from both the donor's liver (wild) and from the recipient's liver (control and AdSRαhOTC-infected at different moi's). The recipient was a severely affected OTC-deficient male with an exon 3 TaqI site mutation. Hepatocytes were assayed 6 d after plating (4 d after Ad vector infection). The first lane (wild) represents wild type enzyme activity found in the hepatocyte cultures from the normal donor liver specimen. The second lane (control) represents the enzyme activity found in the patient's OTC-deficient liver specimen cultures uninfected with AdSRαhOTC, and the following lanes represent OTC-deficient cultures infected with AdSRαhOTC at different moi's.
on these data and in view of the δgal in vitro and in vivo experiments, that corrective levels of OTC enzyme activity can easily be achieved in spf mice and ultimately in human OTC-deficient livers. These experiments are ongoing and should be valuable in determining the adenoaviral moi needed to obtain the phenotypic corrective levels of enzyme activity. Since human females heterozygous for the OTC mutation who have ~ 50% OTC activity are asymptomatic, it should not be necessary to achieve wild type levels of OTC activity for phenotypic correction (27, 28). Furthermore, while this paper has focused on hepatic expression; ectopic expression of OTC in the small bowels of transgenic sparse fur mice corrected the urea cycle metabolic defect (2). These features offer many logical approaches to gene therapy "correction" of OTC deficiency. Strong expression of OTC in deficient patients during a hyperammonemic crisis could be of great benefit, even if the expression is transient. Currently available adenoaviral vectors may not support permanent gene expression, because they are not incorporated into the host cell genome. Therefore, the strength of a promoter in such a system may be more relevant than the longevity of the expression. However, for permanent gene therapy, liver-specific or long-term promoters should be of potential benefit.

The safety of adenoaviral vectors is an issue that requires further study. Although the adenoaviral serotypes 4 and 7 have been used for decades as oral vaccines without significant complications, there are still potential hazards (27–30). This issue is particularly critical in terms of assessing the safety of the currently used replication-defective adenoaviruses, since replication-defective adenoaviruses lacking after infection can be partially complemented not only by wild type adenoavirus, but also by heterologous immediate early proteins of other viruses including HCMV and EBV (31–33). Although the liver is a rare target for clinical adenoaviral infection, it can be a target for HCMV (34, 35). Viral vectors deleted in all E1 functions both after infection and E1B, are less likely to manifest leukosis at high mois or be complemented by early functions of heterologous viruses. Nevertheless additional studies are required to evaluate the usefulness of the currently available defective adenoavirus vectors and to determine whether additional alterations may be desirable. It is noteworthy in this context that a vast array of Ad mutants, temperature-sensitive in viral functions, is available to generate vectors which are impaired if necessary.

This study suggests that adenoaviral vectors may provide a powerful tool for transduction of high level gene expression in the liver and moves us one more step toward clinical gene therapy trials. In the case of OTC deficiency, short- and/or long-term expression would have utility.

Acknowledgments

We are grateful to P. Wood and L. Swisher for providing us with the OTC-deficient liver and an unused portion of the donor liver at Texas Children’s Hospital. We thank B. J. F. Rossiter for her valuable assistance in preparing the manuscript; M. Abbodati, P. Clemens, and J. Pearlman for their critical review; and J. Rudy and P. Jones for their technical assistance.

Funding was provided by the National Institutes of Health, C. T. Caskey is also a Howard Hughes Medical Institute Investigator and F. L. Graham is a Terry Fox Research Scientist of the National Cancer Institute of Canada.

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