DNA-Damaging Agents Greatly Increase the Transduction of Nondividing Cells by Adeno-Associated Virus Vectors

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Received 8 July 1994/Accepted 26 September 1994

None of the vector systems currently available for gene therapy applications have been shown to be capable of both efficient gene transfer into nondividing cells and long-term expression through stable integration into host cell DNA. While integrating vectors based on adeno-associated virus are capable of mediating gene transfer into nondividing cells, this process is 200-fold less efficient than transduction of dividing cells. We demonstrate that the transduction efficiency of adeno-associated virus vectors can be increased by treatment with DNA-damaging agents. Nondividing cells are especially responsive, with increases in transduction efficiency of up to 750-fold. This finding has the potential to facilitate gene therapy applications requiring gene transfer to nondividing cells.

Efficient delivery of therapeutic genes to nondividing cells, with resultant long-term expression, remains a major goal in the development of gene therapy. To achieve this goal, new gene delivery systems need to be developed or the performance of existing systems needs to be improved. The retroviral vectors based on Moloney murine leukemia virus used in many current gene therapy protocols, while capable of integration and stable long-term expression, require cell division for efficient transduction (24). In contrast, vectors based on adenovirus (15) and herpes simplex virus (12) are capable of transducing nondividing cells but do not integrate into host cell DNA with any appreciable frequency. Vectors based on adeno-associated virus (AAV) have been shown to integrate (17, 21, 23, 27, 29), but their potential for transducing nondividing cells has yet to be fully investigated.

AAV is a nonpathogenic dependent parvovirus with natural tropism for human cells (19). The viral genome, of approximately 4.7 kb (32), is encapsidated as a single-stranded DNA molecule of either sense (5), and only the terminal repeat sequences, of 145 bases, are required in *cis* for viral replication, packaging, and integration (23, 29). Productive infection usually requires coinfection with adenovirus (1) or herpesvirus (8), which provide helper functions for viral replication. In the absence of helper functions, the AAV genome can integrate into host cell DNA, establishing a latent infection. Vectors based on AAV exploit these features and are attractive alternatives to the vector systems currently used in gene therapy applications (9, 25).

We have recently demonstrated that AAV vectors preferentially transduce cells in S phase of the cell cycle (27). However, transduction events do occur independently of S phase at a 200-fold lower frequency. The factors present within an S-phase cell that facilitate transduction by AAV vectors remain undefined but are likely to directly involve or be closely linked to DNA synthesis. Possible factors include host cell polymerases required for conversion of the single-stranded input genomes to double-stranded molecules and/or cellular factors facilitating vector integration.

While studying the relationship between transduction by AAV vectors and S phase, we noted that the presence of tritiated thymidine increased transduction efficiencies. This observation led us to postulate that induction of DNA synthesis outside S phase might facilitate the transduction of nondividing cells. In the experiments reported here, we explored this hypothesis in stationary cultures of primary human fibroblasts exposed to a panel of DNA-damaging agents. We found that such agents greatly increased the transduction of nondividing and, to a lesser extent, dividing cells. Furthermore, the effect on nondividing cells can be achieved with clinically applicable regimens.

MATERIALS AND METHODS

Cell culture. HeLa cells and 293 cells have been described elsewhere (13, 14). Neonatal primary human foreskin fibroblasts were kindly provided by Christine Halbert (Fred Hutchinson Cancer Research Center) and were isolated as previously described (22). Cells were maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 2.5 µg of amphotericin B per ml at 37°C in a 10% CO₂ atmosphere. Stationary cultures of primary human fibroblasts were prepared in 6-cm-diameter dishes (Corning) as previously described (27) and contained about 2×10^6 cells per dish.

Vector construction, production, and assay. Plasmid pALAPSN, used to generate the AAV vector AAV-LAPSN, has already been described (27). Plasmid pALβgeo, used to generate the AAV vector AAV-Lβgeo, was constructed from plasmid pTR (kindly provided by Sergei Zolotukhin and Nicholas Muzyczka) by using standard molecular techniques. The sequences of pALAPSN and pALβgeo are available on request. Plasmids were propagated in *Escherichia coli* JC8111 (6), and vector stocks were generated as previously described (17, 27). Particle numbers were determined by Southern blot analysis of purified vector DNA as previously described (27, 29). Fibroblast cultures were infected with AAV vector stocks at multiplicities of infection of approximately 1 to 2 particles per cell.

Analysis of alkaline phosphatase and β-galactosidase expression. Histochemical staining for alkaline phosphatase ex-

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pression in individual cells was performed as previously described (10). Alkaline phosphatase activity in cell lysates was determined by a spectrophotometric assay as previously described (3), with minor modifications. Cell lysate was prepared as previously described (4), and 10 to 100 μ l of lysate was brought to a final volume of 100 μ l with homogenization buffer and diluted with 100 μ l of 2 $\times$ reaction buffer (2.0 M diethanolamine [pH 8.0], 1.0 mM $MgCl_2$). Twenty microliters of *p*-nitrophenylphosphate (120 mM in 1 $\times$ reaction buffer) was added to initiate the assay. The optical density at 405 nm was then monitored as a function of time. Controls included a blank with no cell lysate and cell lysates from uninfected cultures. The protein content of each sample was determined by using the method of Bradford (7). For analysis of β -galactosidase expression, cells were washed once with phosphate-buffered saline (PBS), fixed in 3.4% formaldehyde in PBS, washed three times with PBS over 30 min and then stained in 5 mM $K_3Fe(CN)_6$ -5 mM $K_4Fe(CN)_6 \cdot 3H_2O$ -2 mM $MgCl_2$ -1 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside per ml in PBS for 8 h at 37°C.

Physical and chemical treatments. Cells were exposed to gamma radiation at 364 rads/min from a ^{137}Cs source in an M38-1 Gammator (Radiation Machinery Corporation) and UV (254 nm) irradiated with a Stratilinker UV cross-linker (Stratagene) immediately before vector addition. [*methyl*- 3H]thymidine (82 to 89 Ci/mmol; Dupont-NEN) was incubated with experimental cultures from the time of vector addition until analysis of alkaline phosphatase expression 48 h later. Cultures were preincubated with *cis*-platinum(II)-diammine dichloride (Sigma) for 16 h and then washed twice with fresh medium prior to vector addition. Cultures were treated with nocodazole (Sigma) from 1,000-fold-concentrated stocks dissolved in dimethyl sulfoxide from 2 h prior to vector addition until analysis of alkaline phosphatase expression 48 h later. Cultures were treated with methotrexate (Sigma) from 100-fold-concentrated stocks dissolved in 50 mM $NaHCO_3$. Cultures were preincubated with methotrexate for 20 h and then washed twice with fresh medium prior to vector addition.

Autoradiography. Tritium labeling and analysis of S-phase cells were performed as previously described (27).

DNA manipulations and analysis. Isolation of episomal AAV vector DNA from stationary-phase cultures was performed by using a modification of the Hirt procedure (18, 27). Southern blot analysis was performed by using standard procedures (28).

RESULTS

DNA-damaging agents increase AAV vector transduction efficiency. We chose stationary primary human fibroblast cultures to study the effects of DNA-damaging agents on the transduction efficiency of AAV vectors. These cultures contain a predominance of nondividing cells, with only 4% passing through S phase each day, and allow the relative transduction efficiencies of AAV vectors on these subpopulations to be studied. Transduction was defined as the process encompassing both gene transfer and expression. The effects of UV light (254 nm), gamma irradiation, *cis*-platinum, and tritiated thymidine on transduction were determined by examining the relative numbers of cell foci expressing alkaline phosphatase in treated and untreated cultures 48 h after exposure to the vector AAV-LAPSN (Fig. 1). Each agent increased the transduction efficiency of AAV-LAPSN (Fig. 2). At the maximum doses tested, the increases ranged from 20- to 90-fold. The effect was not vector specific, as gamma irradiation also

FIG. 1. Maps of the AAV-LAPSN and AAV-L β geo vector genomes. AAV-LAPSN contains the Moloney murine leukemia virus promoter (MLV) (33), the human placental alkaline phosphatase gene (AP) (20), the simian virus 40 early promoter (SV) (26), and the neomycin phosphotransferase gene (*neo*) (2). AAV-L β geo contains the Moloney murine leukemia virus promoter and fusion gene β geo, which encodes both β -galactosidase and neomycin phosphotransferase activities (11). The positions of the inverted terminal repeat sequences (ITR) and the polyadenylation signal (pA) are indicated. Transcriptional promoters are indicated by arrows.

increased the transduction efficiency of a second vector, AAV-L β geo (Fig. 1) (data not shown).

Two cytotoxic agents that do not directly damage DNA were also tested: nocodazole, an inhibitor of mitotic spindle formation, and methotrexate, a folic acid antagonist. At nocodazole concentrations ranging from 50 ng/ml to 1 μ g/ml, all of which produced mitotic arrest, there was little or no effect on transduction efficiency (data not shown). Similarly, methotrexate had no effect on transduction efficiency over the concentration range of 10^{-4} to 10^{-9} M (data not shown). These results are consistent with the conclusion that DNA damage or associated events play an important role in the phenomenon reported. The failure of nocodazole to block transduction also

FIG. 2. Effects of four agents on the transduction efficiency of AAV-LAPSN on stationary primary human fibroblast cultures. (A) tritiated thymidine; (B) UV light at 254 nm; (C) *cis*-platinum; (D) gamma irradiation. Dark and light shading indicates data from two independent experiments. Relative transduction efficiency was calculated by dividing the number of cell foci expressing alkaline phosphatase in treated cultures by the number present in untreated cultures. A relative transduction efficiency of 1 is equivalent to 54 ± 16 focus-forming units per plate (mean $\pm$ standard error of the mean; $n = 8$).

FIG. 3. Effect of gamma irradiation on the relative transduction efficiency of AAV-LAPSN on S-phase and non-S-phase cells in stationary primary human fibroblast cultures. Cells were gamma irradiated with doses ranging from 250 to 4,000 rads 8 h prior to addition of 10 μ Ci of tritiated thymidine per ml and the vector AAV-LAPSN. After a further 48 h, the total number of transduction events was determined by staining for alkaline phosphatase expression and the proportion of transduction events occurring independently of the S phase was determined by autoradiography. The 8-h interval between irradiation and vector exposure was employed to reduce the proportion of dividing cells in the cultures. Relative transduction efficiencies were calculated by using values obtained from a control culture that received both the vector and tritiated thymidine but no gamma irradiation. A relative transduction efficiency of 1 was equivalent to 165 and 32 focus-forming units per plate for S- and non-S-phase populations, respectively.

supports our earlier conclusion that mitosis is not required for transduction by AAV vectors (27).

Enhanced transduction of nondividing cells. In two independent cultures treated with 10 μ Ci of tritiated thymidine per ml (Fig. 2A), it was possible to determine which of the cells expressing alkaline phosphatase had been transduced independently of both S phase and mitosis by coating the stained monolayers with a nuclear emulsion and performing autoradiography. Cells expressing alkaline phosphatase with tritium-labeled nuclei represent the population that were in S phase at some point during the period in which transduction occurred. By using this technique, we were able to determine that 10% of transduction events had occurred independently of both S phase and mitosis (27). Since the presence of 10 μ Ci of tritiated thymidine per ml increased transduction 18-fold (mean), the number of transduction events occurring independently of both S phase and mitosis exceeded the total number of transduction events occurring in control cultures that did not receive tritiated thymidine by 1.8-fold. Thus, the presence of tritiated thymidine must have increased the transduction of nondividing cells by greater than 1.8-fold. However, the magnitude of the increase in nondividing cell transduction could not be determined by using autoradiography alone because the percentage of non-S-phase cells transduced in control cultures was unknown.

To further define the effects of DNA-damaging agents on the transduction of nondividing cells, we combined autoradiog-

TABLE 1. Effect of gamma irradiation on alkaline phosphatase expression from a G418-selected polyclonal population of primary human fibroblasts transduced with AAV-LAPSN^a

Treatment group (radiation dose [rads])	Alkaline phosphatase activity (nmol of substrate hydrolyzed/ min/ μ g of protein)	
	Culture 1	Culture 2
Control naive fibroblasts	<0.001	<0.001
Transduced fibroblasts (0)	0.46	0.42
Transduced fibroblasts (4,000)	0.29	0.29

^a Alkaline phosphatase activity in cell lysates from duplicate cultures in each of three treatment groups was determined 48 h after exposure to 4,000 rads of gamma radiation.

raphy with gamma irradiation (Fig. 3). The increase in transduction efficiency of non-S-phase cells ranged from 7-fold at 250 rads to more than 100-fold at 4,000 rads. The effect of irradiation on the transduction efficiency of S-phase cells was apparent only at radiation doses of 2,000 rads and above. When these data were recalculated by using values obtained from a control culture receiving no tritiated thymidine, increases in transduction efficiency of up to 750-fold were obtained for non-S-phase cells. These higher-fold increases in transduction efficiency represent the combined effects of tritiated thymidine and gamma irradiation and are based on the assumption that 10% of the transduction events in the control culture occurred independently of the S phase. The true increase is likely to be greater than 750-fold, as the 10% value probably overestimates the transduction of non-S-phase cells in control cultures since this value was obtained from experiments requiring tritiated thymidine, which, on the basis of our gamma irradiation data (Fig. 3), would have preferentially increased the transduction of non-S-phase cells.

Increased expression does not account for increased transduction. Increased expression from transductants following exposure to DNA-damaging agents is a potential explanation for our results. To exclude this possibility, polyclonal cultures of G418-selected primary human fibroblasts transduced with AAV-LAPSN were exposed to 4,000 rads of gamma radiation, and 48 h later, their alkaline phosphatase expression was compared with that of unirradiated cultures. There was no difference between the numbers of cells expressing alkaline phosphatase in irradiated and unirradiated cultures (data not shown). Quantitative analysis of alkaline phosphatase activity revealed that irradiation caused a modest fall in alkaline phosphatase expression (Table 1). These data show that the increased transduction efficiency of AAV-LAPSN following irradiation is not due to increased gene expression from pre-existing transcription units.

Episomal vector DNA amplification does not account for increased transduction. Several groups have reported that genotoxic stress, including UV irradiation at 254 nm and exposure to chemical carcinogens, results in helper virus-independent amplification of wild-type AAV DNA (30, 34, 35). One group reported greater-than-400-fold amplification of vector DNA in CHO-K1 cells treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 30-fold amplification in E6 human diploid fibroblasts (35). To determine whether a similar phenomenon might accompany the increased transduction efficiency of AAV vectors in cells exposed to DNA-damaging agents, we examined Hirt supernatants from both irradiated and unirradiated cultures of stationary primary human fibroblasts 48 h following vector exposure. Southern analysis revealed no evidence of DNA amplification

FIG. 4. Autoradiograph of low-molecular-weight DNA isolated from triplicate cultures of stationary-phase primary human fibroblasts in each of the following treatment groups: uninfected cultures, unirradiated cultures infected with AAV-LAPSN, and cultures infected with AAV-LAPSN after exposure to 4,000 rads of gamma radiation. Hirt supernatant DNA was harvested at 48 h for Southern analysis by using a probe specific for vector *neo* sequences. The position of the single-stranded (SS) monomer form of the vector genome is indicated. A phosphorimager was used to quantitate the total hybridization signal in each lane and the signal representing the single-stranded monomer forms of vector DNA. The variation between lanes was within the range of experimental error. Staining of a fourth culture in each group for alkaline phosphatase expression demonstrated that in this experiment, gamma irradiation increased transduction efficiency in excess of 100-fold (data not shown).

in gamma-irradiated cultures (Fig. 4). On much longer exposures (2 weeks versus 4 h in Fig. 4), presumed double-stranded and replicative forms were observed but again there was no difference between irradiated and unirradiated cultures (data not shown). These data demonstrate that the increased transduction efficiency of AAV vectors in irradiated cells is not associated with significant amplification of episomal vector DNA. Analysis of high-molecular-weight DNA in the Hirt pellets for integration events by PCR was not possible because of residual low-molecular-weight AAV vector DNA contamination of this fraction.

Reporter gene expression persists over time. The number of cells expressing alkaline phosphatase in gamma-irradiated (4,000 rads) stationary-phase cultures was monitored for 28 days after infection with AAV-LAPSN. The number of transduced cells increased 14-fold between 2 and 28 days postinfection (Fig. 5). The increases in the number of cells expressing alkaline phosphatase in irradiated cultures above that in unirradiated cultures were 53- and 78-fold at 2 and 9 days postinfection, respectively. At 9 days postirradiation, there was no appreciable difference in viability between irradiated and unirradiated cells as determined by trypan blue exclusion. However, the ability of gamma-irradiated fibroblasts to form colonies was reduced by 50% at 450 rads, and at doses of 2,000 rads and above, colony formation was not observed (data not shown).

DISCUSSION

We have demonstrated that the transduction efficiency of AAV vectors on nondividing cells can be increased by more than 2 orders of magnitude by treatment with DNA-damaging agents and that the transduction of dividing cells can be

FIG. 5. Increase in the number of transduced cells over time in cultures of irradiated primary human fibroblasts exposed to AAV-LAPSN. The vector AAV-LAPSN was added to cultures immediately following exposure to 4,000 rads of gamma radiation, and cultures were stained for alkaline phosphatase expression at various intervals for up to 28 days.

increased by up to 20-fold. Our initial hypothesis was that AAV vector transduction requires DNA synthesis and that induction of unscheduled DNA synthesis by exposure to DNA-damaging agents might facilitate transduction of nondividing cells. The demonstration that DNA-damaging agents increase the transduction efficiency of AAV vectors on stationary primary human fibroblasts is consistent with this hypothesis. However, since tritiated thymidine and gamma irradiation also increased the transduction of S-phase cells, it is unlikely that the effect is due simply to increased levels of DNA synthesis. Whatever constitutes the naturally permissive environment in S-phase cells can be further modulated by DNA-damaging agents. Damaged DNA might provide sites for integration or, alternatively, lead to induction of repair enzymes, including DNA polymerases, and factors modulating cell cycle progression that increase AAV vector transduction.

Although we have not defined the mechanism underlying the effect reported, we have excluded increased expression from stable transductants and episomal DNA amplification as possible explanations. Instead, increased transduction appears to reflect an increase in the number of input vector genomes converted to a form that allows gene expression. The relationship between gene expression and integration has not been established for AAV vectors. It is conceivable that vector gene expression could occur in the absence of integration from input vector genomes that have undergone second-strand synthesis but remain episomal. However, we have previously noted that the infectious titer of vector AAV-LAPSN on primary human fibroblasts determined by G418 resistance or alkaline phosphatase expression is equivalent, suggesting that alkaline phosphatase expression can be equated with stable gene transfer. Furthermore, as all of the G418-resistant colonies examined contained integrated vector sequences (27), transduction and integration appear to be closely related events. DNA-damaging agents might therefore increase AAV vector transduction by facilitating integration.

We previously observed an increase in the number of

transduction events over time and attributed this effect to transduction by residual vector genomes in the small proportion of cells transiting S phase each day (27). The same explanation cannot be invoked to account for the progressive increase in vector transduction efficiency observed in fibroblast cultures that have received 4,000 rads of gamma irradiation, since such cultures do not contain actively replicating cells. The most likely explanation is that radiation induces a state of enhanced cellular permissiveness for AAV vector transduction and that persistence of this state over time allows recruitment of vector genomes to occur.

AAV vector transduction of primary cells, including primary skin fibroblasts and primary human airway epithelial cells, is much less efficient than transduction of immortalized cell lines (15a). Indeed, in an earlier study, we found that 700 to 5,000 particles per cell are required to transduce 17 to 28% of primary human fibroblasts in vitro (27). Even higher multiplicities of infection are likely to be required in gene therapy applications directed at nondividing cells, particularly when in vivo delivery is necessary. Since higher transduction efficiencies translate into lower particle-to-infectivity ratios, the approach described in this report may be critical to the practical implementation of gene therapy protocols employing AAV.

Increases in AAV vector transduction efficiency can be achieved on nondividing cells at irradiation doses that do not impair cell viability and have a minimal effect on clonogenic capacity, indicating that this modality has the potential to be applied clinically in either in vivo or ex vivo protocols. In addition, the treatments we employed were not optimized, and modification of treatment regimens should further increase the efficacy of this approach, possibly allowing dividing cells to be targeted. For example, combining different agents might produce synergistic effects, and reduced rates of irradiation and dosage fractionation would allow higher cumulative doses to be used while maintaining cell viability and mitotic potential (16). Higher doses could be employed on relatively radioresistant targets such as liver, muscle, and central nervous system cells, while lower doses could be used on relatively sensitive targets, such as bone marrow stem cells, which must retain mitotic potential (31). Finally, if the effect of DNA-damaging agents on AAV vector transduction efficiency is mediated by a factor(s) induced by DNA damage rather than DNA damage itself, it may be possible to induce this factor(s) with less-cytotoxic agents.

Our findings add a new dimension to the gene transfer technologies currently available for gene therapy applications, in particular, the prospect of effectively targeting nondividing cells with an integrating vector system.

ACKNOWLEDGMENTS

We thank S. Zolotukhin and N. Muzyczka for generously providing bacterial strain JC8111, adenovirus type 2, and plasmids pTR and pTRAAVneo. We also thank C. L. Halbert, D. D. Koeberl, and D. G. Miller for helpful discussions and comments on the manuscript.

This work was supported by grants HL41212 and DK47754 (both to A.D.M.) and CA09645 (D.W.R.) from the National Institutes of Health and by a grant from the Cystic Fibrosis Foundation. I.E.A. is the recipient of a Neil Hamilton Fairley fellowship from the Australian National Health and Medical Research Council.

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