Adenovirus-mediated Augmentation of Cell Transfection with Unmodified Plasmid Vectors

(Received for publication, June 6, 1992)
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The present study demonstrates that the human adenovirus (Ad) can augment transfer and expression of a gene within plasmid DNA unmodified by nonspecific linkers or by linker-ligand complexes. Following the transfection of COS-7 cells with pRSVL, a luciferase expression plasmid vector directed by the Rous sarcoma virus-long terminal repeat promoter, luciferase activity in the target cells was 10^2- to 10^3-fold higher when the cells were also infected with Ad-CFTR, a replication-deficient recombinant Ad containing human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA. The enhancement of luciferase gene expression in COS-7 cells was also observed with Ad-dl312 (a replication-deficient E1a deletion mutant Ad with no exogenous gene) and wild type Ad5. The efficiency of cell transfection with pRSVL in the presence of an Ad was achieved in a dose-dependent fashion with progressively higher luciferase activity in cells infected by increasing amounts of Ad-CFTR, Ad-dl312, or Ad5. The augmentation by Ad-CFTR of the transfer and expression of the luciferase gene in cells was similar to that of another transfection reagent, cationic liposomes. Further, when Ad-CFTR and liposomes were used in combination, 4- to 100-fold more efficient expression of the luciferase gene was achieved than with Ad-CFTR or liposomes alone. When COS-7, HeLa, and CV-1 cells were evaluated in parallel in the presence or absence of liposomes, Ad-mediated enhancement of luciferase activity was observed in all cell lines. Thus, exposure of target cells to replication-deficient or competent human Ad will markedly augment transfer and expression of the genes within plasmid DNA in mammalian cells in vitro without modifying the plasmid with linkers or linker-ligand complexes, a strategy that should be useful for in vitro and in vivo gene transfer applications.

DNA virus (1, 2), is a natural example of a highly efficient means to transfer biologic materials to target cells (3-13). The Ad accomplishes this by fibers on its outer surface interacting with specific cell surface receptors and the subsequent internalization of the virus into the cells (1, 2, 14). Human adenoviruses can infect a broad range of cell types from a variety of species, are easy to prepare in high titer, and can be easily rendered replication-deficient, thus preventing the virus from taking over, and eventually destroying, the target cell (1, 2, 9, 11, 12).

Capitalizing on its ability to easily enter cells, the Ad has been employed as a vehicle to transfer a variety of non-viral macromolecules to target cells in vitro and in vivo. First, the Ad can transfer non-viral macromolecules packaged within the Ad in place of normal Ad components. For example, the genome of the Ad can be modified to include exogenous DNA. The recombinant Ad is then packaged into an infectious virus capable of entering cells and transferring the exogenous DNA to the nucleus, where it is expressed and translated to produce an exogenous protein (9, 11, 12, 15). Second, the Ad can mediate the transfer of non-viral macromolecules either linked to the surface of the Ad or in a “bystander” process where the macromolecule is cointernalized in the Ad receptor-endosome complex (9-8). In terms of general applicability, the cointernalization strategy is very appealing, as it does not require modification of the biologic material to be transferred with linker or ligand.

Based on the knowledge that macromolecules including proteins, carbohydrates, and toxins can be cointernalized with adenoviruses into target cells without modifying the molecule in any fashion (3, 4, 6-8), we hypothesized that it should be possible to augment the transfer of and subsequent expression of genes within plasmid DNA in a similar fashion, i.e. without linking the plasmid to the Ad or to any other biologic materials. In this regard, the present study is designed to evaluate effectiveness of replication-deficient and competent adenoviruses to augment the transfer and expression of a reporter gene in a plasmid vector without linking any biologic reagent to either the Ad or the plasmid.

MATERIALS AND METHODS

Cell Cultures—The Ad type 5 (Ad5) transformed human embryonic kidney cell line 293 (American Type Culture Collection (ATCC), CRL 1573) (16) and the human cervical carcinoma cell line HeLa (ATCC CCL2) were grown in improved minimal essential medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from Biofluids). COS-7, an SV40 virus transformed African green monkey kidney cell line (ATCC CRL 1561) and the non-transformed African green monkey kidney cell line CV-1 (ATCC CCL70) were maintained in Dulbecco’s modified Eagle’s medium (Whittaker Bioproducts) supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Adenovirus and Plasmid Constructs—The replication-deficient recombinant adenoviruses used were Ad-CFTR (based on Ad5, with a deletion of E1a and part of E1b and E3, and containing a 4.5-kilobase human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA driven by the Ad2 major late promoter) (11) and the E1a deletion virus Ad-dl312 (17). The replication-competent Ad was wild type Ad5. All viruses were propagated in 293 cells, recovered 36 h after infection by 5 cycles of freezing/thawing, purified by CsCl density centrifugation (18), dialyzed, and stored in virus dialysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 10% glycerol) at -70 °C prior to use. Titers of the viral stocks were determined by
plaque assay using 293 cells (16, 18). The pRSVL plasmid vector containing the Rous sarcoma virus-long terminal repeat promoter and a firefly luciferase cDNA as a reporter gene was used for all studies (19).

**Transfection and Infection of Cells**—For transfection of COS-7, HeLa, and CV-1 cells, the cells were first detached by trypsin, counted, and 1 × 10^5 cells were seeded in 10-cm plates. After 20 h, cells were washed once with a serum-reduced medium (OPTI-MEM I reduced serum medium Gibco/BRL) and maintained in the OPTI-MEM I medium for 2 h until transfection. Unmodified pRSVL plasmid was then added to the cultured cells. After 30 min, Ad, either Ad-CFTR, Ad-d312, or Ad5, was added to the plates and incubated in culture medium by gentle rocking. Cells were incubated at 37°C in 10% CO2 for 24 h, the medium was aspirated and changed to Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, and the cells were incubated for another 24 h before evaluating luciferase activity. To evaluate the augmentation effect of the Ad on transfection and expression of the luciferase reporter gene in COS-7 cells, 200 plaque-forming units (PFU)/cell of Ad-CFTR were applied to cells following addition of 0–15 μg of the pRSVL plasmid. To investigate a dose-response relationship between the amount of Ad administered and luciferase expression, 0–2000 PFU/cell of Ad-CFTR, Ad-d312, or Ad5 were added to the COS-7 cells exposed to the pRSVL plasmid vector (15 pg) as described above. Further, to compare the efficiency of Ad infection for augmentation of uptake and expression of the gene within the plasmid vector to a known transfection reagent, expression of the luciferase reporter gene in COS-7 cells infected with the pRSVL plasmid together with cationic liposomes (Lipofectin Reagent, Bethesda Research Laboratories) was evaluated along with cell lysate protein, plus cationic liposomes followed by Ad-CFTR infection (200 PFU/cell) (20). Liposomes (0–100 μg) were mixed with 15 μg of pRSVL in a total volume of 100 μl according to the manufacturer’s protocol and added to COS-7 cells. Cells were evaluated for luciferase activity after 48 h of transfection in the absence or presence of the Ad-CFTR infection. COS-7, HeLa, and CV-1 cells evaluated in parallel were transfected with 15 μg of pRSVL in the presence or absence of liposomes (50 μg) and Ad-CFTR (200 PFU/cell).

**Detection of Luciferase Activity**—Luciferase activity in cultured cells was evaluated as described by de Wet et al. (19). After 48 h of the initial exposure of cells to pRSVL (alone or in combination with Ad and/or liposomes), cells were detached from the plates with trypsin, washed twice with phosphate-buffered saline (pH 7.4; Whittaker Bioproducts), and the cell lysate extracted in 200 μl of lysis buffer (100 mM potassium phosphate, pH 7.8, and 1 mM dithiothreitol (Sigma)) by 3 cycles of freezing/thawing followed by centrifugation at 14,000 rpm for 5 min (19). Luciferase activity was assayed with 100 μl of pRSVL cell lysate supernatant, 5 mM ATP (Pharmacia LKB Biotechnology Inc.), 15 mM MgCl2, and 1 mM D-luciferin potassium (Analytical Luminescence Laboratories) using a Monolight 2010 luminometer (Analytical Luminescence Laboratories) (19). The total protein concentration of the target cells was measured by the Bradford method (21) Bio-Rad) with bovine serum albumin as a standard. Luciferase activity was expressed as relative light units (RLU)/μg of cell lysate protein after subtracting background (19).

**RESULTS**

Luciferase activity was detected at low levels in COS-7 cells after addition of pRSVL alone and increased about 6-fold with increasing amounts of the plasmid (Fig. 1). In contrast, COS-7 cells infected with Ad-CFTR following the addition of pRSVL demonstrated a marked increase in luciferase activity. While pRSVL alone yielded a maximum of <15 RLU/μg of cell lysate protein, the addition of Ad-CFTR resulted in 10^6 RLU/μg with only 0.1 μg of pRSVL plasmid, and 10^7 RLU/μg with 15 μg of pRSVL, an increase of 10^4-fold over addition of the plasmid alone. Adenovirus infection itself did not induce luciferase expression by the cells, since COS-7 cells infected with Ad-CFTR alone (in the absence of pRSVL) showed no luciferase activity.

To determine whether Ad-mediated augmentation was specific to COS-7 cells, two additional cell lines were evaluated (Table I). Parallel evaluation of COS-7, HeLa, and CV-1 (non-transformed African green monkey kidney cells) cells showed enhancement of luciferase activity by Ad-CFTR in all three cell lines. The enhancement among the cell types was in the range of 150- to 2000-fold.

The augmentation of expression of the luciferase gene was not specific to the exogenous CFTR gene contained in Ad-CFTR, since augmentation was observed with other adenoviruses including the E1a deletion mutant Ad-d312 as well as wild type Ad5 (Fig. 2). Importantly, for Ad-CFTR, Ad-d312, and Ad5, the augmentation of the expression of the luciferase reporter gene in COS-7 cells was observed in a dose-dependent fashion. When no Ad was added (i.e. pRSVL alone), cells had base-line luciferase activity. However, with increasing amounts of Ad added (20 to 2000 PFU/cell), luciferase activity increased progressively. Strikingly, COS-7 cells infected with 2000 PFU/cell of each Ad demonstrated luciferase activity 10^4-fold higher than when no Ad was added.
Data shown are the average and standard error of the mean.

Luciferase activity after 48 h was expressed as relative light units/μg of cell lysate protein. Shown are data from cells exposed to increasing amounts (0 to 2000 PFU/cell) of Ad-CFTR (●), Ad-d8312 (■), or Ad5 (○) following exposure to a fixed amount (15 μg) of pRSVL. Data shown are the average and standard error of the mean of triplicate determinations.

As previously described (20), exposure of the cells to cationic liposomes together with pRSVL augmented transfection of COS-7 cells in a dose-dependent fashion (Fig. 3). With increasing amounts of liposomes added to the cells with a fixed amount of pRSVL (15 μg), luciferase activity in COS-7 cells increased markedly starting from the basal level with no liposomes and reaching a level of 10^4 RLU/μg cell lysate protein with 50 and 100 μg of cationic liposomes. Cells incubated with liposomes alone did not demonstrate luciferase activity (not shown). Interestingly, the level of luciferase activity achieved by Ad-CFTR infection plus pRSVL (Fig. 2) was similar to the level of luciferase activity achieved by transfection with pRSVL plus 50 μg of liposomes (Fig. 3). Importantly, the combination of exposure to plasmid plus liposomes and the subsequent Ad-CFTR infection demonstrated even greater reporter gene expression than exposure of the cells to pRSVL with either liposomes or Ad-CFTR alone. This 4- to 100-fold augmentation (depending on the amount of liposomes added) was achieved in a dose-dependent fashion with increasing luciferase activity using increasing amounts of cationic liposomes. The combined effect of liposomes and Ad-CFTR was observed in HeLa and CV-1 cells as well (Table I). The enhancement among all cell types was in the range of 10^2- to 10^4-fold.

**DISCUSSION**

The adenovirus is a non-enveloped double-stranded DNA virus that enters cells through an efficient receptor-mediated endocytosis pathway (1, 2, 14). The outer shell of each Ad is comprised of 240 hexons and 12 pentons. Each penton is composed of a penton base and a fiber; the fiber is responsible for the initial virus-receptor interaction (1). Following attachment, the receptors with bound Ad cluster in coated pits, and the virus is internalized within a clathrin-coated vesicle and subsequently into an endocytic vesicle (termed endosome or receptosome) (4). The pH of the endosome is reduced via a proton pump associated with the endosomal membrane, the reduced pH alters the conformation of Ad capsid proteins, the endosome is disrupted, and the endocytic contents, including the Ad, are released into the cytoplasm (4, 5, 14, 22-27).

The ability of the Ad to easily enter cells can be used to transport macromolecules into cells (9-13). For DNA, it has been assumed that the exogenous DNA must be inserted into the Ad, are released into the cytoplasm (4, 5, 14, 22-27). The ability of the Ad to easily enter cells can be used to transport macromolecules into cells (9-13). For DNA, it has been assumed that the exogenous DNA must be inserted into the Ad, are released into the cytoplasm (4, 5, 14, 22-27).

**Fig. 2.** Evaluation of luciferase activity in COS-7 cell lysates after exposure to pRSVL and increasing amounts of adenovirus. Luciferase activity after 48 h was expressed as relative light units/μg of cell lysate protein. Shown are data from cells exposed to increasing amounts (0 to 2000 PFU/cell) of Ad-CFTR (●), Ad-d8312 (■), or Ad5 (○) following exposure to a fixed amount (15 μg) of pRSVL. Data shown are the average and standard error of the mean of triplicate determinations.

**Fig. 3.** Comparison of luciferase activity in COS-7 cell lysates after exposure to pRSVL and increasing amounts of cationic liposomes in the absence or presence of adenovirus. Luciferase activity after 48 h was expressed as relative light units/μg of cell lysate protein. Increasing amounts of liposomes (from 0 to 100 μg) were incubated with a fixed amount of pRSVL (15 μg) and then added to the cells. Shown are data from cells not exposed to Ad-CFTR (○) or cells subsequently infected with 200 PFU/cell Ad-CFTR (●). Data shown are the average and standard error of the mean of triplicate determinations.
of enhancement in COS-7 compared with CV-1 is due to an interaction of the adenovirus with the SV40 components of the cells cannot be determined by these data. In the least, because the HeLa cells show similar to greater enhancement the adenovirus can be achieved with a replication-deficient recom-

diplasmid DNA into target cells. Since a similar level of enhance-

ments complex cluster on the membrane and are internalized (4–8). These processes are not Ad-specific, as similar phenomena have been observed with other non-enveloped viruses such as picornavirus (3,7,29,30), as well as enveloped viruses including paramyxovirus, rhabdovirus, poxvirus, and togavirus (3,7,29,30). The cointernalization process is dependent on the multiplicities of infection, occurs early (within minutes) after exposure of the target cell to the virus, and does not require a replication-competent virus. Most attention on virus-mediated cointernalization of macromolecules has been focused on cointernalization of proteins, including toxins and various reporter proteins (3–5,7,8,28–30). The concept that cointernalization might be used for nucleic acids was suggested, but not evaluated, by Otero and Carrasco (7). The present study, consistent with a number of studies with Ad-mediated cointernalization of proteins and carbohydrates (3–8,28–30), dem-

onstrates that modification of the plasmid is not a prerequi-
site, i.e., that like other macromolecules, Ad infection of target cells results in efficient cointernalization of unmodified plasmid DNA into target cells. Since a similar level of enhancement of transfer and expression of the foreign gene in the plasmid can be achieved with a replication-deficient recombinant Ad as with wild type Ad, there is no reason to use wild type Ad, thus eliminating harmful adverse effects such as cell death.

Independent of the mechanism involved, the observations in the present study clearly demonstrate that adenoviruses markedly enhance the transfer and expression of the genes within unmodified plasmid DNA in target cells in vitro. Although there may be a limitation in Ad-mediated augmentation among different cell types due to the number of Ad receptors on the cell surface, this is a simple, efficient means with the potential for a broad variety of applications to transfer nucleic acids to cells in vitro and in vivo. /n

Acknowledgments—We thank A. Pavirani (Transgene, Strasbourg, France), and M. Perricaudet and L. D. Stratford-Perricaudet (Institut Gustave Roussy, Villejuif, France) for providing the original Ad-CFTR construct; T. E. Shenk (Princeton University) for the gift of Ad-d312; H. S. Ginsberg (Columbia University, New York) for the gift of Ad-2303; E. M. Scherer for technical assistance; and T. Horion for helpful discussion.

REFERENCES


