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ARTICLE

Free Cholesterol Enhances Adenoviral Vector Gene Transfer and Expression in CAR-Deficient Cells[1]

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Efficient adenovirus vector-mediated gene transfer depends on the presence of sufficient amounts of the high-affinity coxsackie–adenovirus (Ad) receptor (CAR) on the surface of the target cell leading to receptor-mediated endocytosis of the vector. The present study evaluates the effect of free cholesterol, a lipid component of endocytic vesicles, on Ad uptake into CAR-deficient cells. Infection in the presence of free cholesterol at its maximum solubility in water led to increased binding, uptake, and expression of Ad in human skin fibroblasts and alveolar macrophages, two primary human cells known to be deficient in CAR. The effect of free cholesterol was maximal at its solubility maximum in aqueous solution. Increase of Ad vector-mediated gene transfer with cholesterol was dependent on the lack of CAR receptor expression on the surface and was diminished by overexpression of CAR in CAR-deficient cells. Cholesterol-mediated increase of Ad-mediated gene expression was dependent on coincubation of both cholesterol and Ad and was not dependent on the cholesterol content of the cell. Increased Ad vector-mediated gene expression in the presence of free cholesterol was also observed in murine skin *in vivo.* Structural analysis of the Ad–cholesterol mixture showed complexation between Ad particles leading to formation of multivirus aggregates due to hydrophobic interaction. The addition of free cholesterol with Ad vectors may be a simple way to increase Ad-mediated gene transfer to cells that are poor targets due to their lack of a sufficient number of Ad receptors.

INTRODUCTION

Replication-deficient recombinant adenovirus (Ad) vectors efficiently transfer genes to a variety of cell types *in vitro* and *in vivo* (6, 36, 45). Cell entry of the Ad is mediated by the binding of the Ad fiber to the high-affinity coxsackie–adenovirus fiber receptor (CAR) and the binding of the penton base to lower affinity cell surface integrins (3, 39, 42). Cells that do not express CAR, such as fibroblasts, smooth muscle cells, and macrophages, are poor targets for Ad gene transfer (17, 19, 20, 43, 44).

Strategies to increase Ad transfer into Ad receptor-deficient cells have included upregulation of the expression of α_V integrins on the cell surface (7, 19), overexpression of CAR in the target cells (17, 20, 25), and modification of the binding properties of Ad, such as engineering positively charged domains in the fiber protein to increase the affinity of virions to negatively charged cell-surface molecules such as heparan sulfates (43). Other strategies to increase Ad gene transfer include using Ad together with lipid particles such as cationic liposomes, cationic polymers, and surfactant, all of which increase Ad uptake into Ad receptor-deficient cells mainly by non-receptor-mediated uptake mechanisms (2, 5, 8, 9, 28, 32, 40, 46). In studies using lipids to enhance Ad uptake, mixtures of various lipid components have been used, some of which contain a component cholesterol (9). Cholesterol is an integral part of the plasma membrane where it plays a role in structure and function (37) of membrane protein and bilayer fluidity. Ninety percent of free cholesterol within the cell is found in the plasma membrane from where it exchanges with different intracellular cholesterol pools (22). Free cholesterol is highly lipophilic and has a low solubility in water. Due to its low aqueous solubility, cholesterol is transported in the circulation within li-

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poproteins, small amounts as free cholesterol within the phospholipid membrane, and the majority as cholesterolesters within the core (4, 14).

The present study focuses on the effects of coincubation of free cholesterol with Ad to enhance gene transfer and expression efficiency in Ad receptor-deficient cells. The study demonstrates that mixing of free cholesterol with Ad prior to infection leads to increased Ad gene transfer in CAR-deficient primary human skin fibroblasts with no effect in CAR-expressing cells. The maximum effect was obtained at the maximum solubility concentration for free cholesterol. The cholesterol-mediated increase of Ad expression was associated with increased uptake of the Ad, was blocked by the addition of anti-Ad specific antibodies, and was not dependent on cellular cholesterol content. Finally, increased Ad transfer was observed in mouse skin *in vivo* following intradermal administration. Together, these results demonstrate that mixing free cholesterol with Ad leads to the formation of cholesterol–virus aggregates that exhibit enhanced uptake of Ad. This might be a useful means to increase Ad-mediated gene expression in Ad receptor-deficient cells.

METHODS

Cell culture. Primary human dermal fibroblasts (HDF-1) were obtained from a normal volunteer (17). Alveolar macrophages were obtained from normal volunteers by bronchoalveolar lavage (20). Both cell types were maintained in "complete medium" [RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin (all components from GIBCO BRL, Gaithersburg, MD)]. The A549 cell line (CCL185: American Type Culture Collection, Rockville, MD) was cultured in DMEM, supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Adenoviral vectors. The recombinant Ad vectors used in this study are E1a-, partial E1b-, and partial E3- vectors based on the Ad5 genome. The expression cassette in the E1 position includes the cytomegalovirus early/intermediate promoter/enhancer (CMV), an artificial splice signal, the transgene, and a SV40 poly(A) stop signal (16). The vectors include Adβgal, containing the *Escherichia coli* β-galactosidase (βgal) gene (16), AdCAR, containing the human CAR cDNA (17, 20), and AdNull with no transgene (16). For the binding studies, Adβgal was labeled with carbocyanine dye Cy3 (Amersham Life Sciences Inc., Arlington Heights, IL) or with fluoroisothiocyanate (FITC) as previously described (26). The vectors were propagated and purified, and activity (in plaque-forming units, pfu) was determined, as previously described (34, 35).

Increase of Ad transfer using cholesterol. To evaluate if free cholesterol would increase Ad-mediated gene expression, 3 μg/ml cholesterol or 0.5 μg/ml 25-OH cholesterol (a derivative of cholesterol; both from Sigma, St. Louis, MO) was mixed with serum-free medium containing Adβgal at 37°C and incubated for 30 min at 37°C. The stock solutions, dissolved in 100% ethanol, were 4 mg/ml for cholesterol and 2 mg/ml for 25-OH cholesterol. Infections in the absence of cholesterol were always performed using an equal addition of ethanol. The presence or absence of ethanol had no effect on Ad-mediated gene transfer (not shown). Skin fibroblasts (CAR deficient) (17), alveolar macrophages (CAR deficient; 20), or A549 cells (CAR expressing) (17, 20) were infected with the cholesterol/Ad mixture for 60 min (10 m.o.i. for fibroblasts and 1 m.o.i. for A549 cells). Following infection, the cells were washed three times with serum-free medium and complete medium was added to continue the culture for 48 h. The cells were then lysed and βgal activity was determined in the cell lysates with a luminometer using the Galacto-Light kit (Tropix Inc., Bedford, MA) and adjusted for total cellular protein using the BCA assay (Bio-Rad Laboratories Inc., Hercules, CA). The limit of detection was 10^3 relative light units (RLU)/mg protein.

Dose dependence of free cholesterol-mediated increase of Ad-mediated expression. To determine the optimal dose of free cholesterol to increase Ad transfer in CAR-deficient cells, fibroblasts or the control A549 cells were infected (60 min) with Adβgal (10 m.o.i. for fibroblasts, 1 m.o.i. for A549 cells) mixed with free cholesterol at a dose range from 0.5 to 10 μg/ml and the cholesterol/Ad mixture. βgal activity was determined 48 h following infections as described above.

Ad genome in the cells following addition of cholesterol to Ad. To determine if cholesterol-mediated enhancement of Ad gene expression was a result of increased transfer of the Ad genome into the infected cells, fibroblasts were infected with Adβgal at a dose of 50 m.o.i. mixed with free cholesterol (3 μg/ml) for 60 min. The cells were subsequently washed three times with serum-free medium and collected by the addition of trypsin (0.5 mg/ml; GIBCO/BRL Life Technologies, Inc., Gaithersburg, MD) after 0 and 24 h. Total cellular DNA was extracted using the QIAamp blood kit (QUIAGEN, Inc., Santa Clara, CA). The AdE2 region and β-actin gene were amplified and quantified by TaqMan PCR (30). A fluorogenic probe (CCGCCTCGGCTTGTCACATTTTTC), designed to anneal to the target between the sense primer (AATAAACAAGTTCCCGGATCGAT) and antisense primer (GCACATAGGAGAGATGAGCTTCC) specific for the DNA binding protein sequence in the E2 region of the Ad genome, was used. Samples were amplified for 40 cycles in a Perkin–Elmer 7700 sequence detection system with continuous monitoring of fluorescence. Data were processed by the SDS 1.6 software package (Perkin–Elmer). For human β-actin detection, β-actin probes (Perkin–Elmer) were used.

Cell association and subcellular localization of Ad mixed with cholesterol. To evaluate the cell association and binding of Ad mixed with free cholesterol, fibroblasts were infected with FITC–Ad in the presence of 1 or 4 μg/ml of free cholesterol for 30 min. The cells were then washed, carefully scraped from the wells, resuspended, and washed three times in phosphate-buffered saline, pH 7.4 (PBS). The amount of FITC–Ad associated with the cells was determined by flow cytometry.

To visualize the subcellular localization of the Ad following infection in the presence of cholesterol, fibroblasts were grown on 35-mm culture plates that were modified by punching a 7-mm hole in the bottom and resealing with a coverglass to create a well for the cells to be plated directly on the coverglass. Fibroblasts were infected with Ad conjugated with Cy3 fluorescent dye (Amersham Life Science) at a dose of 10^4 particle units (pu/cell) for 30 min in the presence or absence of 3 μg/ml cholesterol (26, 30). Cells were washed to remove unbound vector and were either fixed immediately in 4% paraformaldehyde in PBS (23°C, 15 min) or incubated for an additional 60 min prior to fixation. Nuclei were stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) in PBS with 0.1% Triton X-100 for 5 min. The coverglass was detached from the culture plate, mounted on a glass slide with antifade solution (Slowfade; Molecular Probes), and evaluated by fluorescence and differential interference microscopy using a Nikon Microphot SA microscope and 60× N.A. 1.4 objective.

Effect of cholesterol depletion on Ad-mediated gene expression. To analyze the effect of depletion of cellular cholesterol on free cholesterol enhancement of Ad-mediated gene expression, human fibroblasts were incubated in complete medium for 16 h with 250 μM mevalonate and 10 μM compactin, followed by incubation with 10 mM cyclodextrin for 30 min to decrease cellular cholesterol. Following cholesterol depletion, the cells were infected with Adβgal (10 m.o.i.) in the presence or absence of 3 μg/ml cholesterol as described above. β-Galactosidase activity was measured 48 h following infection in the cell lysates.

CAR overexpression in human fibroblasts. To evaluate the effect of CAR overexpression on cholesterol-mediated increase of Ad-mediated gene expression, fibroblasts were infected with AdCAR (10 and 100 m.o.i.). Uninfected cells or cells infected with AdNull were used as controls. After 2 days, the cells were infected with Adβgal in the presence of cholesterol as described above and the βgal expression was measured 48 h following the second infection.

Effect of neutralizing antibodies on cholesterol-mediated increase in Ad-mediated gene expression. To study the effect of blocking neutralizing antibodies on cholesterol-mediated increase in Ad-mediated gene expression in fibroblasts, the fibroblasts were infected with Adβgal in the presence of 3 μg/ml cholesterol and serial dilutions of human serum containing high amounts

of Ad specific neutralizing antibodies (anti-Ad titer 1:6400). The serum was obtained from an individual who had received an E1–E3- Ad gene transfer vector ($Ad_{GV}CD.10$) by intrabronchial administration as part of a clinical study and subsequently developed systemic anti-Ad5 neutralizing antibodies, as determined by neutralizing antibody assay (13). The antibody and Ad–cholesterol mixtures were incubated for 30 min at 37°C before infection of the cells. β-Galactosidase activity was determined after 48 h as described above.

Ad-mediated gene expression with cholesterol in vivo. To evaluate if cholesterol would enhance Ad-mediated gene expression *in vivo*, Adβgal (2×10^7 pfu) mixed with cholesterol was administered subcutaneously to C57Bl/6 mice. Anesthetized mice were injected with the Adβgal cholesterol mixture subcutaneously on the dorsal skin using a 30-gauge needle (injection volume 20 μl). Seventy-two hours following administration, the mice were sacrificed, and a 1 × 1-cm skin piece was excised around the injection site, homogenized, and evaluated for βgal-activity as described above.

Structural analysis of the Ad–cholesterol mixture. To determine the structure of Ad in the presence of cholesterol, both fluorescence microscopy (using the fluorophore-labeled virus) and negative-stain transmission electron microscopy were used. For the fluorescence microscopy study, Adβgal labeled with carbocyanine dye Cy3 (Amersham Life Sciences Inc.) was incubated with cholesterol (3 μg/ml) as described above and analyzed by fluorescence microscopy (30). For the electron microscopic investigation. Adβgal was incubated with cholesterol (3 μg/ml) as described above. Specimens were prepared by applying 5 μl of sample onto a carbon-coated grid, washing with aqueous phosphotungstic acid (PTA) 1% wt/wt, and blotting with filter paper. Grids were viewed with a JEOL 100 CX-II electron microscope at 80 kV.

Statistical analysis. All data are presented as means ± standard error of the mean; statistical evaluations were carried out using the two-tailed Student *t* test.

RESULTS

Cholesterol-Mediated Increase of Ad-Mediated Gene Expression in Fibroblasts

To evaluate if the addition of free cholesterol will influence Ad-mediated gene expression *in vitro*, human primary skin fibroblasts (CAR-deficient) or A549 cells (CAR-expressing) were infected with Adβgal in the presence or absence of free cholesterol or 25-OH cholesterol and transgene expression was evaluated 2 days following infection. Expression of the βgal transgene was increased in fibroblasts infected in the presence of cholesterol ($P < 0.0001$) or 25-OH cholesterol ($P < 0.001$) compared to controls treated with the same amount of diluent (ethanol; Fig. 1A). However, in the CAR-expressing A549 cells, the addition of cholesterol or 25-OH cholesterol had no effect on transgene expression ($P > 0.7$, compared to controls; Fig. 1B).

To determine the optimal dose of free cholesterol on the increase of Ad-mediated gene expression in CAR-deficient cells, human fibroblasts or A549 cells were incubated with Adβgal in the presence of increasing doses of free cholesterol ranging from 0.5 to 10 μg/ml. The expression of βgal in the fibroblasts infected in the presence of cholesterol was increased at all doses used, with a maximum sevenfold increase at 3 μg/ml compared to no cholesterol ($P < 0.001$, for all doses compared to controls; Fig. 2A). Interestingly, no increase in transgene expression was observed in A549 cells with any of the doses of free cholesterol used ($P > 0.2$, all groups; Fig. 2B).

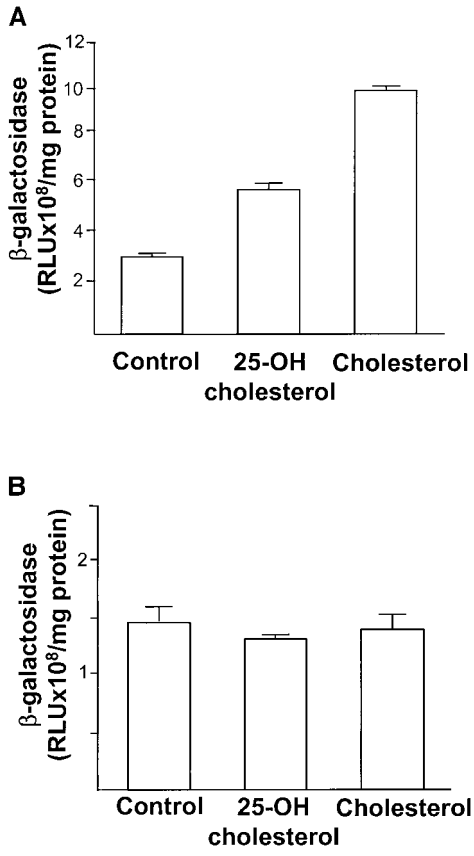

Fig. 1. Ability of cholesterol to increase Ad-mediated gene transfer in human fibroblasts. Cells were infected with Adβgal in the presence of free cholesterol (3 μg/ml) or 25-OH cholesterol (0.5 μg/ml) for 60 min; infection with Adβgal in the presence of ethanol served as a control. β-Galactosidase activity was evaluated 48 h following infection. (A) Human fibroblasts (10 m.o.i.). (B) A549 cells (1 m.o.i.). Each data point represents the mean ± standard error of triplicate measurements.

Effect of Cholesterol on Enhanced Ad Uptake

To evaluate if the increase in Ad transgene expression was associated with an increase in the amount of Ad sequence in the cells, the amount of Ad genome in the fibroblasts was quantified using TaqMan PCR following infection with Ad mixed with free cholesterol. Fibroblasts which were infected with cholesterol showed increased amounts of Ad genome present immediately after infection ($P < 0.01$), as well as 24 h following infection ($P < 0.0001$; Fig. 3). The increased amounts of genome present in the cells at both time points were similar ($P > 0.1$).

The cell association and binding of Ad infection in the presence of cholesterol in fibroblasts were evaluated by using fluorescent-labeled FITC–Adβgal. Fibroblasts infected with FITC-labeled Ad showed a small, but significant increase of the amount of FITC–Ad associated with the cells (mean fluorescence of infected cells 0.46 ± 0.01 compared to 0.37 ± 0.02 for uninfected cells, $P < 0.01$; Figs. 4A and 4B). However, cells infected in the presence of 1 or 4 μg/ml cholesterol showed an enhanced amount of FITC–Ad associated with the cells (mean fluorescence

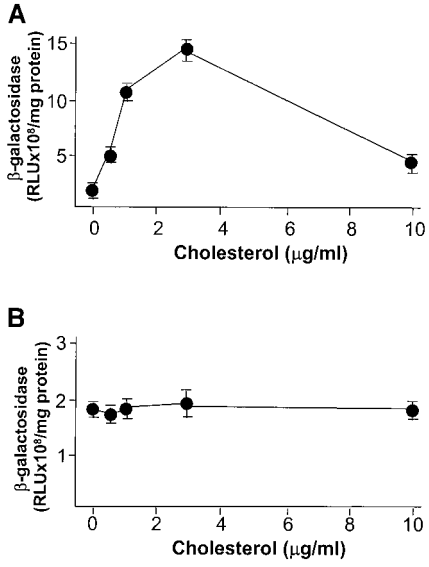

Fig. 2. Dose dependence of cholesterol-mediated enhancement of Ad-mediated gene transfer in human fibroblasts. Fibroblasts were infected with Adβgal in the presence of free cholesterol (0.5–10 μg/ml) for 60 min; infection with Adβgal in the presence of ethanol served as a control. β-gal activity 48 h following infection in (A) fibroblasts (10 m.o.i.) and (B) A549 cells (1 m.o.i.) is shown. Each data point represents the mean ± standard error of triplicate measurements.

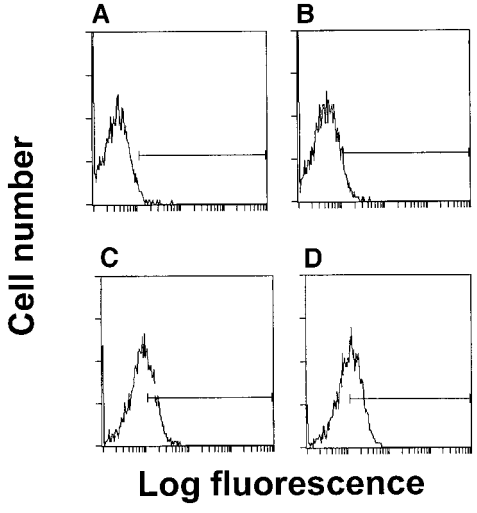

Fig. 4. Increase in cell association and uptake of Ad in the presence of cholesterol. Human fibroblasts were infected with FITC-labeled Ad in the presence of free cholesterol; uninfected cells or cells infected with FITC-labeled Ad in the presence of ethanol served as controls. Sixty minutes following infection, the cells were analyzed for cell-associated Ad by flow cytometry. Cell number is on the ordinate and log fluorescence of the FITC-labeled Ad is on the abscissa. (A) Uninfected fibroblasts; (B) FITC–Adβgal; (C) FITC–Adβgal + 1 μg/ml cholesterol; (D) FITC–Adβgal + 4 μg/ml cholesterol. Representative panels of triplicate measurements are shown.

0.79 ± 0.03 for FITC–Ad + 1 μg/ml cholesterol, 1.02 ± 0.02 for FITC–Ad + 4 μg/ml cholesterol, $P < 0.001$ for both groups compared to FITC–Ad alone; Figs. 4C and 4D).

To assess the subcellular localization of the Ad vector capsid following Ad infection in the presence of cholesterol, human fibroblasts were infected with fluorophore-labeled Cy3–Adβgal in the presence of cholesterol for 30 min with or without a 60-min chase. Compared to control cells, cells infected in the presence of cholesterol showed an increased amount of Cy3–Ad associated with cells as well as an increase in the intensity of fluorescent staining of cell-associated particles, likely indicating aggregation (Figs. 5A–5D). One hour after infection in the presence of cholesterol, cell-associated Cy3–Ad appeared in a dispersed pattern of puncta with uniform intensity (Figs. 5E and 5F). The distribution of Cy3–Ad in some cells showed predominant localization near the nucleus (Figs. 5E and 5F, arrows).

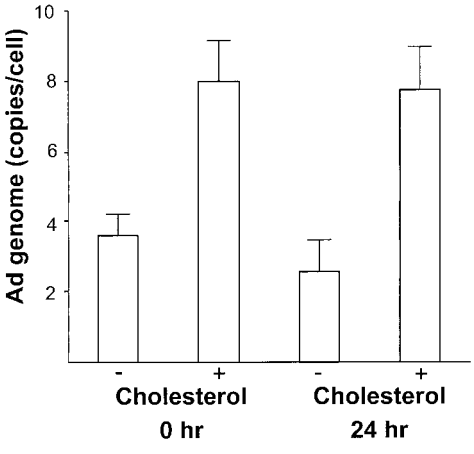

Fig. 3. Cholesterol-mediated increase of the Ad genome in human fibroblasts. Fibroblasts were infected with Adβgal in the presence of free cholesterol (3 μg/ml) for 60 min; infection with Adβgal in the presence of ethanol served as a control. DNA was extracted immediately after or 24 h following infection. The amount of Ad genome was determined by real-time fluorescent PCR (TaqMan) using primers specific for the DNA binding protein sequence in the E2 region of the Ad vector. The data are normalized to genomic DNA. Each data point represents the mean ± standard error of triplicate measurements.

Effect of Pre-/Posttreatment with Cholesterol and Cholesterol Depletion

To evaluate if the addition of cholesterol before, during, or after infection with Ad had similar or different influences on Ad-mediated gene expression, fibroblasts were treated with 3 μg/ml cholesterol pre-, during, or postinfection with Adβgal. βgal expression was increased only when Adβgal was added and mixed with cholesterol (Fig. 6), suggesting that the direct interaction of Ad with cholesterol is necessary for increased Ad-mediated gene expression (preinfection vs control and preinfection vs postinfection, $P > 0.8$; during vs preinfection and during vs postinfection, $P < 0.01$).

To analyze the effect of cellular cholesterol depletion prior to Ad infection in the presence of cholesterol, fibroblasts were depleted of cholesterol using cyclodextrin and compactin and subsequently infected with Adβgal in the presence of cholesterol. As previously seen, the infection

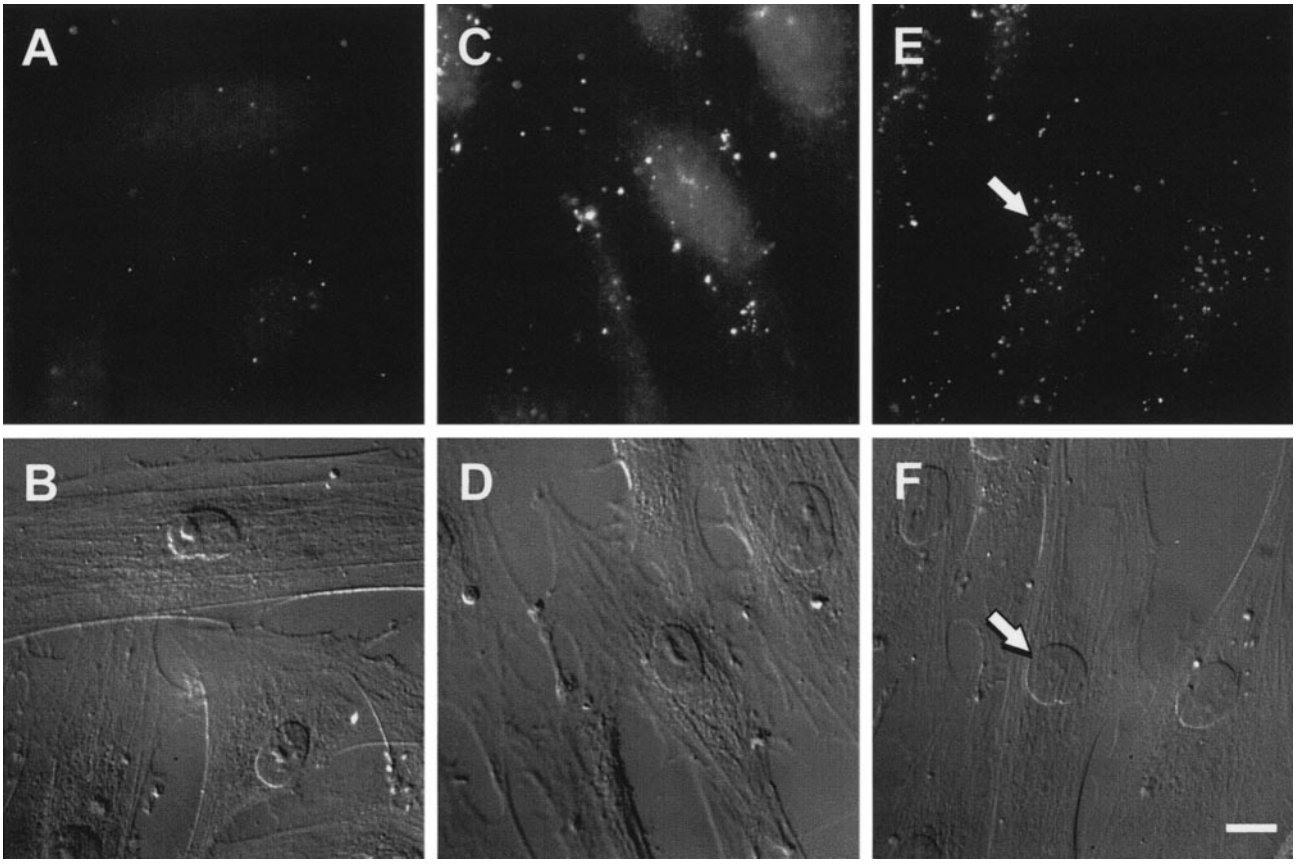

Fig. 5. Subcellular localization of fluorescent Ad in fibroblasts following coincubation with cholesterol. Human fibroblasts were infected with fluorescently labeled Cy3-Adβgal (10^{11} particle units/cell) in the presence or absence of cholesterol (3 μg/ml) for 30 min. Cells were either fixed immediately after infection or incubated for 60 min before fixation. Cells were analyzed by fluorescence and differential interference microscopy. (A) Fibroblasts + Cy3–Adβgal, 30 min infection. (B) Field corresponding to A showing cell morphology. (C) Fibroblasts + Cy3–Adβgal + cholesterol, 30 min infection. (D) Field corresponding to C showing cell morphology. (E) Fibroblasts + Cy3–Adβgal + cholesterol, 30 min infection followed by 60 min incubation. (F) Field corresponding to E showing cell morphology. Arrows indicate nuclear association of Cy3–Ad 60 min after infection. Scale bar = 20 μm.

in the presence of cholesterol led to an increase in transgene expression ($P < 0.005$; Fig. 7). A similar increase was observed in cells depleted of cholesterol ($P < 0.05$). Cholesterol depletion did not influence the extent of transgene expression without or with subsequent addition of free cholesterol with Ad infection ($P > 0.3$, for both comparisons), suggesting that cellular cholesterol did not influence the cholesterol-mediated increase in Ad-mediated gene expression.

Effect of CAR Overexpression

To evaluate if the CAR deficiency of the fibroblasts is an important factor in the cholesterol-mediated increase of Ad-mediated gene expression in the fibroblasts, the fibroblasts were infected with a high dose of AdCAR to express CAR in these cells and then subsequently infected with Adβgal vector in the presence of cholesterol. The cells which received AdNull or were not infected in the first infection showed a significant increase of βgal expression in the presence of cholesterol following the second (Adβgal) infection (all comparisons, $P < 0.005$; Fig, 8).

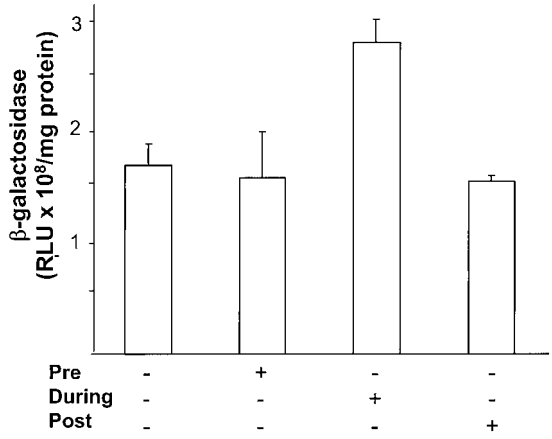

Fig. 6. Dependence on time of addition of cholesterol on cholesterol-mediated increase of Ad-mediated βgal expression in fibroblasts. Skin fibroblasts were infected with Adβgal pre-, during, or post-coincubation with free cholesterol (60 min); infection with Adβgal in the presence of ethanol served as a control. β-Galactosidase activity was determined 48 h following infection. Each data point represents the mean ± standard error of triplicate measurements.

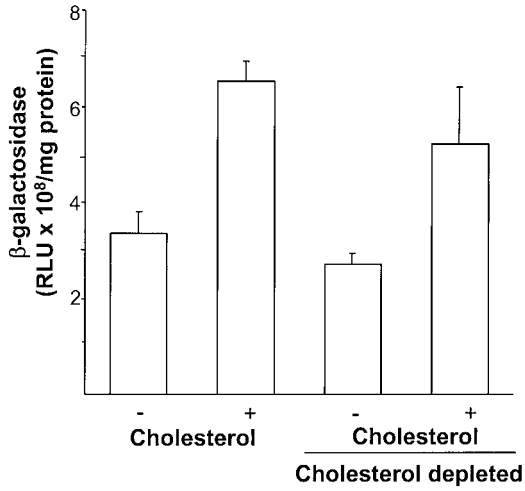

Fig. 7. Effect of cholesterol depletion on cholesterol-mediated increase in Ad-mediated gene expression in human skin fibroblasts. Fibroblasts were depleted of cholesterol by incubation with 250 μM mevalonate and 10 μM compactin for 16 h followed by cyclodextrin (10 mM) for 30 min prior to infection with Adβgal in the presence or absence of cholesterol (3 μg/ml); infection with Adβgal in the presence of ethanol served as a control. β-Galactosidase activity was measured 48 h following infection. Each data point represents the mean ± standard error of triplicate measurements.

Cells that had previously been infected with AdCAR at 100 m.o.i. showed a marked increase in βgal expression compared to uninfected controls and AdNull-infected cells ($P < 0.005$, both comparisons). However, the increase in βgal expression in the presence of cholesterol at the second infection was not significant ($P > 0.2$).

Effect of Neutralizing Antibodies

To evaluate if the cholesterol-mediated increase of Ad-mediated gene expression was through Ad-specific pathways, fibroblasts were infected with cholesterol plus anti-Ad neutralizing antibodies. At the highest concentration of the neutralizing antibodies, the infection was blocked in the absence or presence of cholesterol (Fig. 9). Interestingly, at decreasing doses of neutralizing antibodies, the cells were infectible and the infections with cholesterol showed enhanced expression (titer 1:1000, $P < 0.005$). The blocking effect was attenuated in the presence of cholesterol (1:1000 dilution of serum led to >70% decrease in control βgal expression but only a 40% decrease in βgal expression in the presence of cholesterol), suggesting that cholesterol may sheild Ad from anti-Ad antibodies. However, the overall similarity in the pattern of inhibition suggests that the entry and/or trafficking pathway for the Ad vector + cholesterol is similar, but not identical, to that of Ad alone.

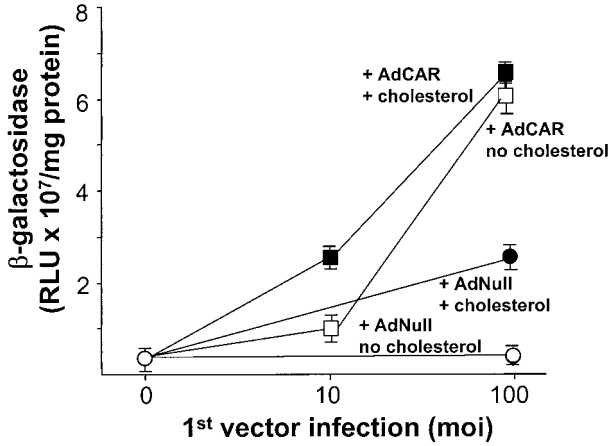

Fig. 8. Effect of overexpression of the Ad high-affinity CAR receptor on cholesterol-mediated increase of Ad-mediated expression in fibroblasts. Skin fibroblasts were infected with AdCAR or AdNull. Forty-eight hours later, the cells were infected with Adβgal in the presence of cholesterol (3 μg/ml); infection with Adβgal in the presence of ethanol served as a control. β-Galactosidase activity was measured 48 h following the second infection. Each data point represents the mean ± standard error of triplicate measurements.

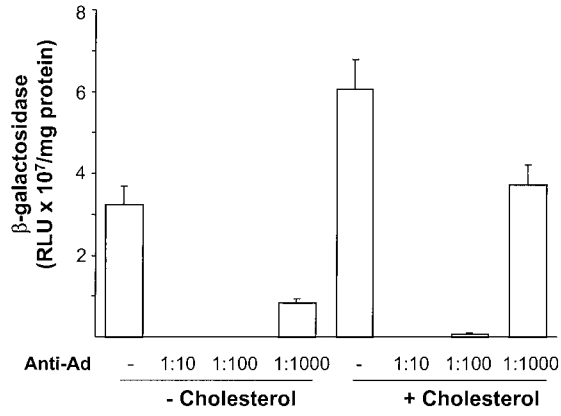

Fig. 9. Effect of neutralizing antibodies on cholesterol-mediated increase of Ad-mediated expression in fibroblasts. Fibroblasts were infected in the presence of various dilutions of neutralizing anti-Ad human serum and free cholesterol (3 μg/ml) for 60 min. β-Galactosidase activity was evaluated 48 h following infection. Each data point represents the mean ± standard error of triplicate measurements.

Ad Transfer in Macrophages

To evaluate if CAR-deficient cells other than human fibroblasts could be infected with increased efficiency with the addition of cholesterol, alveolar macrophages, cells known to be CAR deficient (20), were infected with Adβgal in the presence of free cholesterol (0.5–10 μg/ml). Interestingly, as seen with the fibroblasts (Fig. 2), addition of cholesterol increased Ad-mediated gene expression in a dose-dependant fashion, with a maximum at 3 μg/ml ($P < 0.01$; Fig. 10), i.e., CAR-deficient cells other than fibroblasts can be more efficiently infected by Ad in the presence of cholesterol.

Ad Transfer to Skin in Vivo

Increased expression of βgal was observed in mouse skin following administration of Adβgal mixed with cholesterol compared to Ad alone ($P < 0.01$), suggesting that cholesterol can enhance Ad-mediated gene expression *in vivo* (Fig. 11).

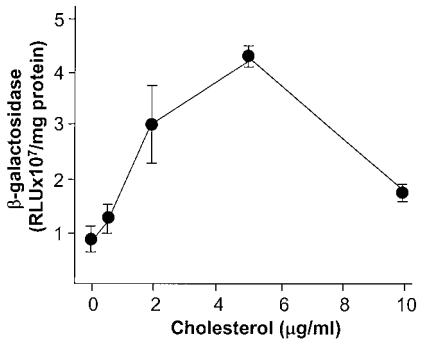

Fig. 10. Ability of cholesterol to enhance Ad-mediated expression in alveolar macrophages. Human alveolar macrophages were infected with Adβgal (25 m.o.i.) for 60 min in the presence or absence of cholesterol (0.5–10 μg/ml); infection with Adβgal in the presence of ethanol served as a control. β-Galactosidase activity was measured 48 h following infection. Each data point represents the mean ± standard error of triplicate measurements.

Structural Analysis of the Ad–Cholesterol Mixture

To characterize the structure of the Ad–cholesterol mixture, Cy3-labeled Ad was incubated in the presence of cholesterol and analyzed by fluorescence microscopy or negative-stain electron microscopy. Mixing of the Cy3–Ad particles with cholesterol molecules led to increased fluorescent particle size compared with Cy3–Ad alone (Figs. 12A and 12B, see white arrows). The preparations were further analyzed using transmission electron microscopy. A comparison of negative-stained samples showed that cholesterol-containing samples exhibited small aggregates of Ad particles bearing electron-lucent coats (Figs. 12C and 12D, see black arrows). Cholesterol-treated Ad capsids often appeared to adhere to one another along edges or facets of the icosahedron. Aggregates of as many as 10 virus particles complexed with cholesterol were observed.

DISCUSSION

Ad vector-mediated gene transfer requires efficient binding and uptake of the Ad by the target cell, and cells that are deficient in the expression of Ad receptors are poor targets for Ad vectors (3, 7, 17, 19, 20, 25, 39, 42). The present study demonstrates that the combination of Ad with free cholesterol leads to increased binding, uptake, and subsequent expression of Ad in CAR-deficient fibroblasts and alveolar macrophages *in vitro* as well as mouse skin *in vivo* following subcutaneous administration. The effect of increased Ad transfer by cholesterol was not dependent on the cholesterol content of the cell at the time of infection and was only effective if cholesterol and Ad were added to the cells simultaneously. Ad transfer was not increased by free cholesterol in CAR-sufficient A549 cells, was diminished by overexpression of CAR in the CAR-deficient fibroblasts, and was partially blocked by anti-Ad neutralizing serum. Structural analysis of the Ad–cholesterol mixture showed complex formation among Ad particles in the presence of cholesterol, based on the hydrophilic interaction. In the context that cholesterol is a simple compound to use as an adjuvant for gene transfer, and that clinical grade cholesterol is readily available, the strategy of mixing Ad gene transfer vector with free cholesterol may be useful in augmenting the efficiency of Ad vector-mediated gene transfer into Ad receptor-deficient cell targets.

Enhancement of Ad Entry

Cell entry of Ad is mediated by the binding of the Ad fiber to CAR and to cell-surface integrins, and perhaps other cell-surface molecules such as HLA class I (3, 18, 39, 42). Cells that are deficient in CAR, such as fibroblasts, endothelial cells, and macrophages, are poor targets for Ad gene transfer and CAR has been shown to be rate limiting for Ad infection (3, 17, 19, 20, 43, 44). Strategies to enhance Ad transfer in Ad-receptor-deficient cells or organs, including the combination of Ad with various lipid particles [cationic liposomes, cationic lipids, and surfactant (dipalmitoylphosphatidylcholine)], as well as polycationic polymers, have been demonstrated to lead to increased Ad transfer (2, 5, 8, 9, 28, 32, 40, 46). Most of these strategies utilize cationic charged molecules to combine with the negatively charged adenovirus particle, leading to charge neutralization of the Ad/particle complex (2, 5, 9, 32, 40, 46).

The mechanisms by which these strategies augment Ad-mediated gene transfer are only partially understood. Combining Ad with cationic liposomes to increase infectivity of Ad infection of vascular smooth muscle cells is believed to be mediated via a fiber receptor/integrin-independent pathway (32). Studies using polycationic polymers and cationic liposomes to enhance Ad vector gene transfer in human airway epithelial cells also show an entry pathway independent of the fiber receptor and via a different pathway than Ad alone (9).

In the present study, the addition of cholesterol to enhance Ad-mediated gene transfer was only apparent in CAR-deficient cells, not in cells that expressed abundant endogenous CAR (A549 cells) or in fibroblasts which were

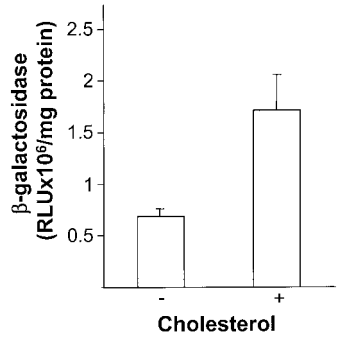

Fig. 11. Cholesterol-mediated increase of Ad transfer in mouse skin. Adβgal (5×10^7 pfu) mixed with cholesterol was injected subcutaneously into the dorsal skin of C57Bl/6 mice (n = 3/group); infection with Adβgal in the presence of ethanol served as a control. β-Galactosidase activity was determined in excised skin sections 48 h following infection.

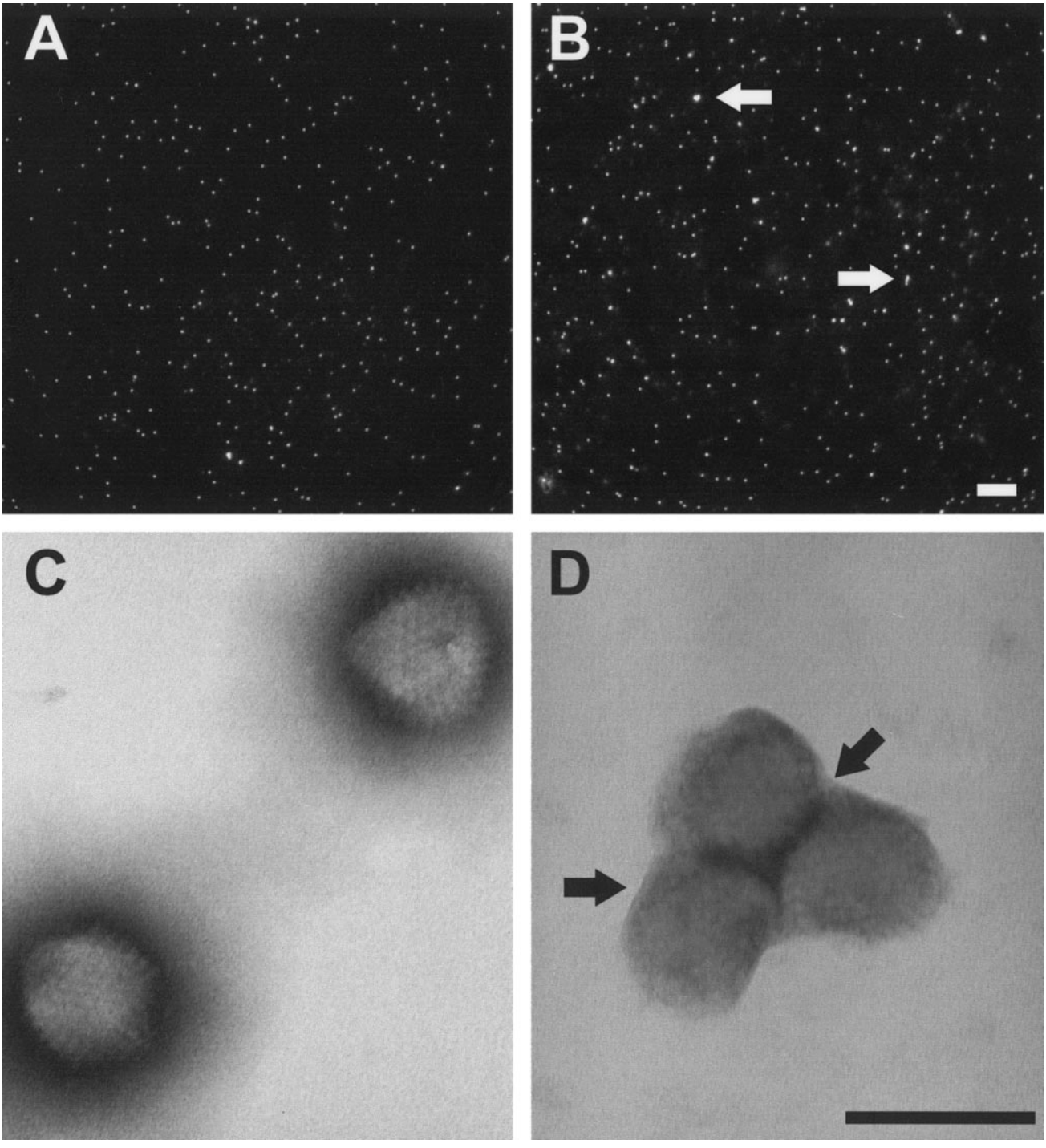

Fig. 12. Structure of adenovirus–cholesterol complex. Adenovirus particles (10^{10} Cy3–Ad particles/ml) were mixed with cholesterol (3 μg/ml) and incubated for 30 min at 37°C. Complexes were analyzed by fluorescence microscopy (A, B) or negative-stain transmission electron microscopy (C, D). (A) Cy3–Ad. (B) Cy3–Ad–cholesterol complexes (white arrows). Scale bar = 10 μm. (C) Phosphotungstic acid (PTA)-stained Ad. (D) PTA-stained Ad–cholesterol complexes with electron-lucent coats (black arrows). Scale bar = 100 nm.

modified to overexpress CAR, demonstrating that the addition of CAR is superior to the addition of cholesterol. Interestingly, anti-Ad neutralizing antibodies blocked the cholesterol-mediated enhancement of Ad gene transfer, although at lower antibody concentration, the blocking effect seemed to lessen in the presence of cholesterol. Since the human fibroblasts used in our study do not express CAR and therefore infection is not blocked at the CAR binding step, this observation suggests that the cholesterol enhancement of Ad vector gene transfer uses the

same intracellular trafficking mechanisms as does Ad vector without cholesterol (26), although the exact pathway(s) of intercellular trafficking need to be studied directly.

Cholesterol Enhancement of Ad-Mediated Gene Transfer

Cholesterol is an integral part of the plasma membrane where it determines structure and function, especially membrane fluidity (37). Ninety percent of free cholesterol within the cell is found in the plasma membrane from where it exchanges with different intracellular cholesterol pools (22). Free cholesterol is highly lipophilic, with a water solubility of only 2 μg/ml (1). Cholesterol does not exist in its free form within the circulation. Rather, plasma proteins transport cholesterol within their phospholipid membranes and as cholesterolesters within their cores (4, 14).

Perhaps because of its hydrophobicity, free cholesterol has not been previously evaluated as a tool for enhancing gene transfer with Ad vector systems. Cationic liposomes and other lipids developed for gene transfer contain cholesterol in various forms as part of their composition (10, 11, 23, 24, 31, 38, 41). In an extensive study comparing the efficiency of various preparations of cationic lipids for gene transfer to the lung, lipids containing cholesterol as a side group were far more efficient than those containing aliphatic or dihydrocholesterol as a side group (23).

As an uncharged particle with a single polar group, cholesterol is not likely to have a strong electrostatic interaction with the charged surface of Ad (27). Structural analysis of the Ad–cholesterol mixture using fluorescence microscopy and electron microscopy demonstrated aggregation of Ad capsids, with some complexes containing as many as 10 Ad capsids. The presence of an electron-lucent coat on cholesterol-treated Ad capsids was unusual given that Ad is known to be a nonenveloped virus (29, 36). The presence of a coat suggested that cholesterol was evenly deposited over the surface of the capsid. The formation of these multivirus aggregates was most likely due to hydrophobic interaction between cholesterol-coated Ad particles.

Cholesterol most likely exerted its effect on Ad-mediated gene transfer through a direct, hydrophobic interaction with the Ad capsid, resulting in the formation of a complex that exhibited increased binding and uptake into the cells. The requirement for cholesterol at the time of Ad infection suggested that a direct interaction of cholesterol with Ad was important. The data demonstrated that intracellular trafficking of vector and DNA persistence were not affected by cholesterol, suggesting that the difference occurred at the point of entry into the cell, although further studies looking at the intracellular trafficking directly need to be done to confirm this. Cholesterol may have influenced the cell membrane in terms of changing fluidity or unmasking molecules or structures which facilitate Ad entry. High local concentrations of cholesterol are associated with inflections of lipid bilayers that precede endocytosis (21, 33). In a study of membrane composition following Ad absorption, marked changes were seen in the number and planar distribution of sterols and intramembranous particles (15), i.e., membrane changes induced by cholesterol could be a possible mechanism for the increased uptake of Ad which usually enters the cell via endocytosis (12, 36). The present study does not implicate changes in cholesterol homeostasis as the mechanism responsible for the cholesterol effect, unless these changes were very rapid and quickly reversible, as preincubation of the cells with cholesterol, as well as cholesterol depletion of the cells using cyclodextrin and compactin, showed no effect on Ad uptake. Future studies should be done to examine the effect of other physiological neutral lipids, especially those that are components of cell membranes, on Ad-mediated gene transfer, to determine whether a common principle governs the effect of these molecules on Ad-mediated gene transfer. In summary, preincubation of Ad with cholesterol may be a simple way to increase Ad-mediated gene transfer to cells that are poor targets due to their lack of a sufficient number of Ad receptors.

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