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Mechanism of adenovirus improvement of cationic liposome-mediated gene transfer

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Abstract

Substantial effort has been focused on the development of highly efficient gene transfer strategies. Although viral and non-viral methods have been elaborated, mechanisms of gene delivery are still poorly understood. We exploited our recent observation that replication-deficient type 5 adenovirus dramatically enhances lipofectAMINE-mediated gene transfer (lipoadenofection) in differentiated cells to elucidate the mechanism of adenovirus action in this process. Heat-induced denaturation of viral capsid abolishes adenovirus action whereas inactivation of viral genome by short treatment with UV has no effect. Electron microscopic observations reveal the formation of a complex containing adenovirus and lipofectAMINE which probably carries DNA into cells via endocytosis. Anti-adenovirus antiserum or monoclonal anti-$\alpha_v\beta_3$ integrin antibody inhibits lipoadenofection, at least partially. Neutralization of endosomal compartments with chloroquine, ammonium chloride or monensin does not prevent adenovirus improvement of gene transfer. Hence, adenovirus–lipofectAMINE–DNA complexes in which viral particles are each encompassed by three lipid layers, penetrate cells via an endocytic pathway involving probably the adenovirus receptor and $\alpha_v\beta_3$ integrin. The resulting efficient transfer and expression of plasmid DNA proceeds from a mechanism in which adenoviral endosomolytic activity appears to be required while viral genome is not essential. © 1997 Elsevier Science B.V.

Keywords: Cationic liposome; Adenovirus; Gene transfer; Adipocyte; Integrin; Endocytosis

1. Introduction

Gene transfer by non-viral methods remains a poorly efficient process, particularly in terminally differentiated cells. Two types of vectors have been widely employed. Soluble DNA-protein complexes utilize the targeting capacity of a ligand that is recognized by a specific receptor at the cell surface and is conjugated to poly-L-lysine to which plasmid DNA is associated [1,2]. Cationic liposomes or polyethylenimine (PEI) allow formation of a condensed structure in which positive charges of the vector interact with negatively-charged phosphate DNA [3–5]. At least in the case of lipid–DNA complexes, receptor-independent endocytosis has been demonstrated as being the major pathway of entry into cells [6,7]. The limiting

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step for efficient gene transfer using non-viral methods is the intra-endosomal DNA breakdown consecutive to fusion of endosomes to lysosomes [7]. With the aim of overcoming this degradation process, the idea has emerged that the endosomolytic property of adenovirus could be exploited.

In the past few years, we and others have developed approaches using the association of replication-deficient adenovirus and either naked plasmid DNA [8], poly-L-lysine–DNA conjugates [2,9–11], cationic liposomes [12–16] or PEI [17] in a covalent [10,11] or non-covalent [2,9,12–17] manner. Such associations actually dramatically enhance gene transfer efficiency in various cell types. Moreover, combination of adenovirus with either lipofectAMINE or PEI has been proved adequate for transfection of plasmid DNA in cultured adipocytes, muscle or hepatocytic cells, three highly differentiated cells almost impervious to efficient transfection by other means [12].

Mechanisms by which adenovirus improves non-viral-mediated gene transfer are poorly documented. It is known however that covalent coupling of adenovirus to poly-L-lysine–DNA conjugates induces formation of a complex which enters cells at least in part through adenovirus receptor-mediated endocytosis [18]. In this report, we have addressed the question of how non-covalent association of adenovirus to cationic liposome–DNA complexes permits a better lipofection efficiency in differentiated cells. We demonstrate that a complex between adenovirus, lipofectAMINE and plasmid DNA penetrates adipose cells via an endocytic process which probably involves the adenovirus receptor and a cellular integrin. We show also that neutralization of endosomal pH is not deleterious to gene transfer enhancement.

2. Materials and methods

2.1. Plasmid and cell culture

The plasmid pSV2-CAT (SV40 promoter fused to the chloramphenicol acetyltransferase (CAT) gene) was described elsewhere [19]. Plasmid was prepared by two successive equilibrium centrifugations in cesium chloride/ethidium bromide gradient. 3T3-F442A adipoblasts [20] were cultured in 60 mm dishes (Falcon) at 37°C in a humidified atmosphere of 10% CO_2/90% air. Cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) containing 200 IU ml^{-1} penicillin, 50 mg l^{-1} streptomycin, 8 mg l^{-1} biotin, 4 mg l^{-1} pantothenate and 3.7 g l^{-1} bicarbonate and supplemented with 10% fetal calf serum. Adipose differentiation was achieved in the same medium supplemented with 0.02 μM insulin for 7 days. Medium was changed every other day.

2.2. Adenovirus propagation and treatment

The replication-deficient recombinant type 5 adeno-virus Ad-RSV-nlsLacZ (Rous sarcoma virus promoter driving the nlsLacZ gene) [21] was propagated in human embryonic kidney cell line 293 (ATCC collection) as previously described [22]. Adenovirus was purified by banding on two successive cesium chloride gradients, dialyzed against phosphate buffered saline containing 10% glycerol, and stored at −80°C. Viral titers were determined by plaque assay using 293 cells. For genome inactivation, adenovirus was exposed to 260 nm UV light source on ice at 4 cm from the lamp, for 1, 2 or 5 min. For alteration of capsid protein, virus was heated at 45°C for 10 min [23].

2.3. Gene transfer procedure

Plasmid DNA (5 μg) and 18.6 μl lipofectAMINE (Gibco BRL) were separately diluted into 50 μl of DMEM in polystyrene tubes, then gently mixed together (10 ± charge ratio). After 10 min, adenovirus (200 plaque forming units (pfu) $cell^{-1}$) was added or not to the lipid–DNA complex prior dilution in 2 ml of serum-free medium which was subsequently added to a 60 mm dish containing ca. 3×10^6 differentiated cells. 7 h later, medium was changed to 4 ml of DMEM containing 10% fetal calf serum. Cells were harvested 16 h later.

2.4. Adenovirus infection and CAT and β-galactosidase assays

Preparation of cell homogenates for CAT assays was performed as detailed elsewhere [12]. The method of Seed and Sheen [24] was used for determination of CAT activity. For measurement of adenovirus infec-

tivity, cells were incubated in 2 ml of culture medium containing 200 pfu cell^{-1} of native or UV light-treated adenovirus for 7 h. β-galactosidase was detected 16 h later either by determination of enzymatic activity as described previously [25] or by staining as follows. Cells were rinsed with phosphate buffered saline, fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside as described [26]. Blue nuclei corresponding to β-galactosidase expressing cells were counted under the microscope 8 h after staining.

2.5. Electron microscopy

Negatively stained material for gene transfer were observed by electron microscopy. Lipid–DNA complexes were achieved with 5 μg of pSV2-CAT and lipofectAMINE in a 1/10 positive charge ratio, then adenovirus was added (200 pfu cell^{-1}) or not. Transfection materials were adsorbed on colodion coated nickel grids, air dried, stained with 0.5% aqueous solution of phosphotungstate and visualised with a transmission electron microscope. Lipofection or lipoadenofection were carried out on 3T3-F442A adipocytes cultured in 35 mm dishes for 30 min, then fixed with 1% glutaraldehyde for 2 h at 4°C. Standard electron microscopy procedure was achieved as previously described [27]. Briefly, cells were post-fixed in 1% osmium tetroxide and dehydrated in graded series of ethanol. Embedded Eponate blocks were sectioned. Sections were stained with uranyl acetate and lead then examined with a transmission electron microscope.

2.6. Neutralization with antibodies

Adenovirus was treated with a polyclonal antiserum from rabbit which had been obtained after three intramuscular immunizations with adenovirus serotype 5 [28]. This antiserum contains neutralizing antibodies directed against proteins of the adenoviral capsid. This serum was used at a dilution of 1/100 for 30 min at 37°C prior addition to the lipid–DNA complex. Pre-immune serum was used under the same conditions as a control. To neutralize $\alpha_v\beta_3$ integrin, cells were treated with hamster anti-mouse monoclonal anti-β_3 IgG (25 μg ml^{-1}) (Pharmingen) in serum-free DMEM for 30 min before proceeding to lipoadenofection.

2.7. Cell treatment with weak bases and ionophores

Differentiated cells were incubated with either 100 μM chloroquine, 10 mM ammonium chloride or 5 μM monensin for 30 min. Then lipoadenofection was carried out in the presence of these agents. For controlling acidification of endosomes, 3T3-F442A adipocytes were pretreated with either 100 μM chloroquine, 10 mM ammonium chloride or 5 μM monensin for 30 min then 1 μM acridine orange was added for 30 min. Acridine orange accumulates in acidic compartments resulting in fluorescent red staining. Cells were immediately observed under microscope with a 546 nm filter.

3. Results and discussion

3.1. Adenovirus capsid but not viral genome is essential for lipoadenofection

Adenovirus binding to the plasma membrane, internalization and endosome-disruption activity are three crucial steps intervening in adenovirus infection [29,30]. Short treatment of adenovirus with UV has been reported to be without effect on adenovirus infectivity, suggesting that viral genome plays no obvious role in this process [31]. We wondered whether UV-treated adenovirus would be efficient for lipoadenofection. We used a complex of pSV2-CAT with lipofectAMINE in a 1/10 positive charge ratio for lipofection of 3T3-F442A adipocytes in the absence or in the presence of either native or UV-treated non-replicative type 5 adenovirus. As expected from our previous findings [12], the presence of adenovirus is highly beneficial to lipofection, with a 50-fold increase in CAT activity (Fig. 1). Lipoadenofection efficiency was entirely conserved at 1 min of treatment of adenovirus with a 260 nm UV light, reduced by about 30% at 2 min and by 60% at 5 min (Fig. 1). In contrast, a 1 min UV-treatment was sufficient to prevent adenovirus infection by 90%, as determined by Ad-RSV-nlsLacZ-mediated β-galactosidase expression (Fig. 1). Longer exposure to UV light totally inhibited infection. The deleterious effect of 1 min UV-treatment on adenovirus infectivity, together with the maintenance of maximal transfer of the plasmid indicated that integrity of the viral genome was not

required for lipoadenofection. This result was in accordance with previous observations showing that UV-treated adenovirus still supported delivery of either naked DNA [32] or transferrin–polylysine–DNA conjugates [31] into cultured cells, while its ability to replicate on 293 cells was obviated.

Next we exposed the adenovirus preparation to 45°C for 10 min. Such a treatment was reported to alter capsid proteins in such a way that a large decrease in adenovirus infectivity [29] and endosomolytic activity [23] was observed. This heat-inactivated adenovirus was used in lipoadenofection experiments. As shown in Fig. 1, CAT activity remained identical to that obtained with lipofectAMINE alone, showing that viral capsid was essential for the enhancement of liposome-mediated gene transfer by adenovirus. A similar dependence of unaltered adenoviral capsid proteins for gene transfer accomplished by the receptor-mediated transferrin–polylysine system has been described already [33].

3.2. DNA, liposomes and adenovirus form a complex that enter cells via the adenovirus-mediated endocytic process

We wondered whether adenovirus would associate with the lipofectAMINE–DNA complex to form a higher ordered complex that would penetrate cells via endocytosis. Electron microscopy observations showed that negatively stained lipofectAMINE–DNA complexes appeared as dense aggregates resembling those observed with other cationic lipids [3,7], although much larger as a consequence of using anionic (aggregating) phosphotungstate instead of uranyle acetate as contrasting agent (Fig. 2A). Adenovirus bound avidly to these condensed structures (Fig. 2B). 3T3-F442A cells were treated for 30 min with a suspension containing the resulting ternary complex, the fate of which was examined by electron microscopy. Condensed structures were found in the vicinity of the cell membrane and in endocytic vesicles holding several lipid-coated adenovirus particles (Fig. 2C–D). Adenovirus-free lipofectAMINE–DNA complexes were also found in endocytic vesicles (not shown) as expected from previous works [7,6]. Periodicity of the lamellar structures was of approximately 5.5–7 nm a size close to that reported using different cationic lipids [7,34], giving rise to multilamellar structures of 70–150 nm (Fig. 2E). In the course of lipoadenofection, each adenovirus particle with a core of 65 nm, was encompassed by 3 lipidic bilayers of identical sizes (3.5–5 nm) separated each by 3 nm and presenting an hexaedric conformation (Fig. 2F). The resulting highly ordered structure had a diameter of 110 nm. In such a structure, the plasmid DNA is likely to be condensed between the lipid bilayers [35].

We next wondered whether the route taken by adenovirus to penetrate cells was of importance for the lipoadenofection process. Adenovirus enters cells by endocytosis after binding to a still unidentified cellular receptor (see *Note* on p. 15) via penton fiber, a protein of the capsid, 37.6 nm in length [36–38]. Internalization requires intervention of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins thought to interact with the RGD sequence of penton base, another protein of the capsid [39–42]. Because of the size of the lipofectAMINE–DNA–adenoviral core complex (see above and Fig. 2F) we postulated that part (about 12 nm) of

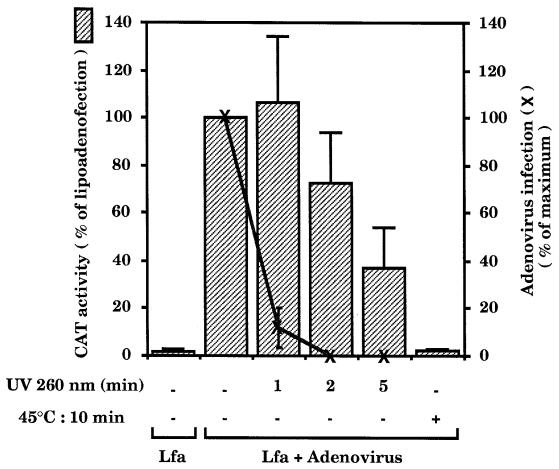

Fig. 1. Role of adenovirus capsid and viral genome for lipoadenofection efficiency. 3T3-F442A adipocytes were transfected for 7 h by lipofection (Lfa) or lipoadenofection (Lfa + adenovirus). Either native adenovirus (200 pfu $cell^{-1}$), or adenovirus treated with UV for 1, 2 or 5 min or with heat (45°C) for 10 min was used or not. After transfection, cells were incubated with serum-containing medium for 17 h before harvesting and determination of CAT activity. Values of CAT activity are expressed in percent of that obtained by lipoadenofection carried out with native adenovirus. Each value represents the mean ± SEM of data obtained from three independent experiments with duplicate dishes. 100% represents 231 ± 61 mU CAT mg $protein^{-1}$.

the penton fibre protruded out of the complex and remained able to interact with the adenovirus receptor (Fig. 3).

For blocking penton fiber, we used a polyclonal adenovirus antiserum previously obtained in immunization experiments [28]. We incubated adenovirus with either this antiserum or the pre-immune counterpart before mixing with the lipofectAMINE–DNA complex and proceeding to lipoadenofection of 3T3-F442A adipocytes. Results are presented in Fig. 4. As expected, adenovirus augmented lipofectAMINE-mediated gene transfer as quantified by CAT activity measurement. Adenovirus anti-serum prevented this increase by 80%, whereas pre-immune serum had no effect. Hence, we could speculate that lipofectAMINE–DNA–adenovirus ternary complex formation did not impair interaction of penton fiber to the adenovirus receptor. Therefore, in our transfection procedure like in those employing either naked DNA [32] or transferrin–polylysine–DNA conjugates [18], adenovirus appeared to use its own receptor for increasing gene transfer efficiency.

For neutralizing $\alpha_v\beta_3$ integrin, we incubated cells with a specific $\alpha_v\beta_3$ monoclonal antibody before lipoadenofection. As shown in Fig. 4, this treatment inhibited by about 40% the adenovirus-mediated increase in CAT activity. Such a partial neutralization was consistent with the potential involvement of a second receptor, the $\alpha_v\beta_5$ integrin, in adenovirus internalisation [41,43]. The mechanism by which the

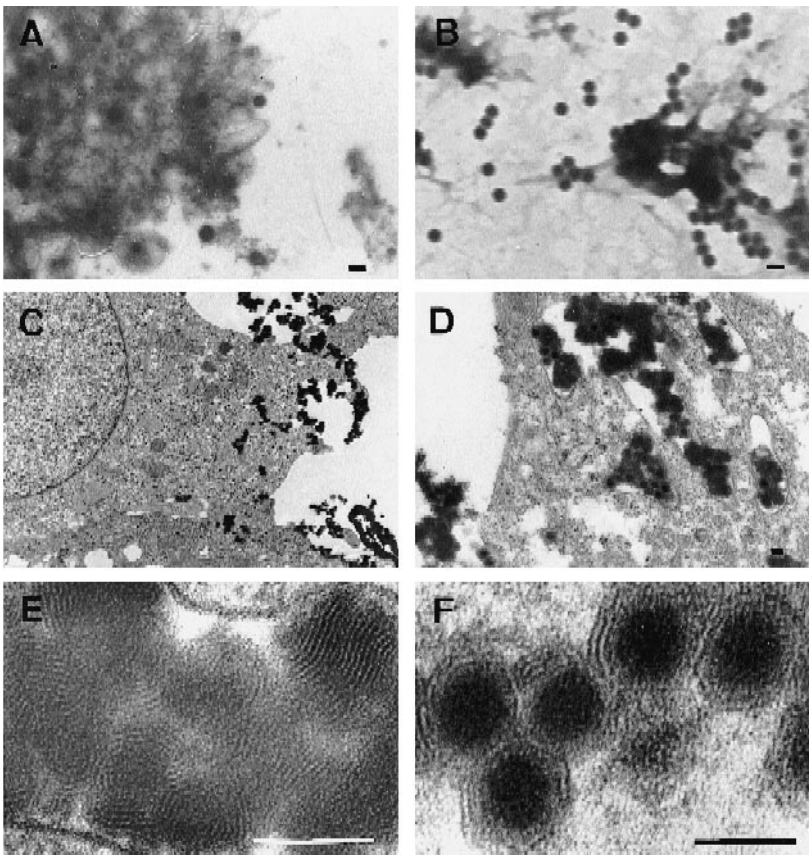

Fig. 2. Electron photomicrographs of gene transfer reagents and of 3T3-F442A adipocytes transfected by lipoadenofection. Lipid–DNA complex was prepared at a charge ratio of 10 + then adenovirus was added or not. These reagents were observed under electron microscope after negative stain or incubated with adipocytes for 30 min before electron microscopy. Negatively stained lipofectAMINE–DNA complex are presented without (panel A) or with adenovirus (panel B). Panels C and D show cells transfected by lipoadenofection, at different magnifications. Panels E and F show endosomes containing lipid–DNA complexes without (panel E) or with adenovirus (panel F). Panels A, B, D, E, F: bar represents 100 nm. Panel C: bar represents 1 μM.

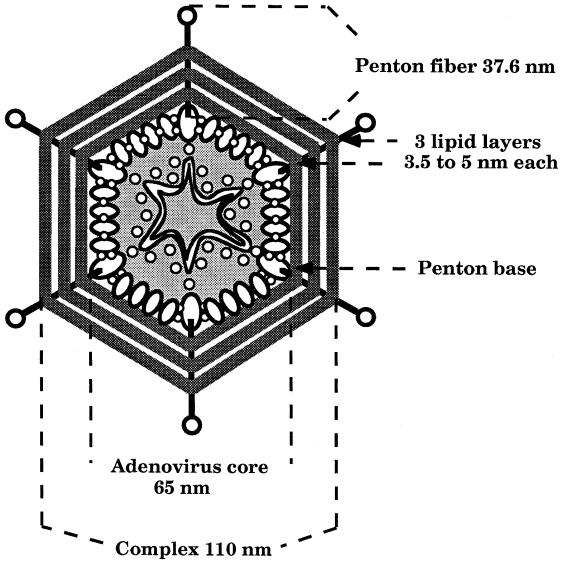

Fig. 3. Schematic representation of the lipofectAMINE–adenovirus complex. Figure drawn from electron microphotographs identical to that of Fig. 2F represents adenovirus encapsulated in 3 lipid bilayers.

RGD sequence of penton base interact with integrins for allowing lipoadenofection to proceed is presently unknown.

Hence, both adenovirus receptor and $\alpha_v\beta_3$ integrin appeared to play a role in the adenovirus-induced increase in lipofection. These results associated to the electron microscopic observations strongly suggested that lipofectAMINE–DNA–adenovirus ternary complexes penetrated cells via receptor-mediated endocytosis, leading to lipoadenofection.

3.3. Neutralization of endosomal pH does not affect lipoadenofection

We wondered whether both components of the vector system, i.e., adenovirus and lipofectAMINE were equally important for plasmid delivery from the endosomal compartment to the cytoplasm. One alternative to dissociate the respective roles of liposome and adenovirus was to neutralize the pH of intracellular compartment. We treated cells with either weak bases (chloroquine, ammonium chloride) or a ionophore (monensin), all agents known to be efficient for neutralizing pH of the intracellular compartments [44,45]. We controlled this neutralizing action by incubating cells for 30 min with these agents then 30 min with acridine orange, a fluorescent molecule able to specifically stain acidic intracellular compartments [46]. Indeed, fluorescent spots were strongly attenuated in cells incubated with the neutralizing agents when compared to those present in acridine orange-only treated cells (Fig. 5).

Cells were transfected by lipoadenofection in the presence or not of either chloroquine, ammonium chloride or monensin and CAT gene expression was measured. A 3.6-fold increase in CAT activity was obtained when chloroquine was used, whereas ammonium chloride or monensin had no effect (Table 1). In contrast, in lipofectAMINE only-transfected cells, reductions of 40%, 75% or 95% in gene transfer were obtained by treatments with respectively chloroquine, ammonium chloride or monensin (Table 1). One interpretation for these results could be that these reductions would be the consequence of a decrease in the endosomolytic potential of dioleoylphosphatidyl

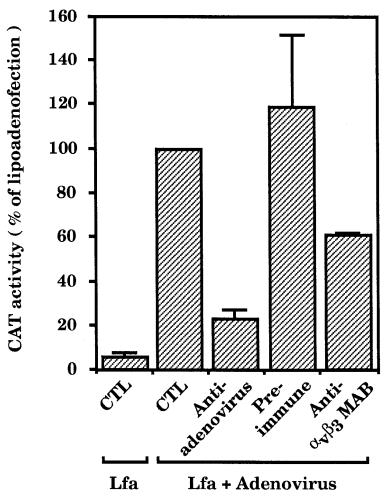

Fig. 4. Effect of anti-adenovirus and anti $\alpha_v\beta_3$ integrin antibodies on lipoadenofection efficiency. 3T3-F442A Adipocytes were transfected as described in the legend to Fig. 1 using either native or neutralized adenovirus (200 pfu cell^{-1}). Adenovirus was treated with either a polyclonal adenovirus antiserum from rabbit or the pre-immune counterpart for 30 min at 37°C. For $\alpha_v\beta_3$ integrin neutralization, adipocytes were pretreated with hamster anti-mouse monoclonal anti-β_3 IgG (Anti-avb3 MAB) for 30 min before proceeding to lipoadenofection. Cells were next incubated with serum-containing medium for 17 h before harvesting and determination of CAT activity. Values of CAT activity are expressed in percent of that achieved by lipoadenofection performed with native adenovirus. Each value represents the mean ± SEM of data obtained from three independent experiments with duplicate dishes. 100% represents 164 ± 57 mU CAT mg protein^{-1}. Lfa, lipofectAMINE; CTL, control.

Table 1
Effect of weak bases and ionophores on transgene expression

	Transgene expression (% of control)			
	Control	Chloroquine (100 μM)	Ammonium chloride (10 mM)	Monensin (5 μM)
Lipoadenofection	100	357 ± 43	108 ± 9	99 ± 32
Lipofection	100	64 ± 14	24 ± 10	4.4 ± 1.2
Infection	100	145 ± 17	99 ± 2	114 ± 18

3T3-F442A adipocytes were pretreated with either 100 μM chloroquine, 10 mM ammonium chloride or 5 μM monensin for 30 min, then lipoadenofection (LipofectAMINE 10+ associated with Ad-RSV-nlsLacZ, 200 pfu cell^{-1}), lipofection (LipofectAMINE 10+) or infection (Ad-RSV-nlsLacZ, 200 pfu cell^{-1}) were carried out for 7 h. 17 h later cells were harvested and CAT (lipofection, lipoadenofection) or β-galactosidase (infection) activities were determined. Values of transgene expression are expressed in percent of that of control cells. Each value represents the mean ± SEM of data obtained from three independent experiments with duplicate dishes. 100% CAT activity represents 227 ± 70 mU mg protein^{-1} and 11 ± 3 mU mg protein^{-1} for lipoadenofection and lipofection, respectively. 100% β-galactosidase activity represents 374 ± 36 mU μg^{-1} h^{-1} for adenovirus infection.

ethanolamine (DOPE) present in lipofectAMINE, when pH is raised to neutral as reported [47]. Interestingly, β-galactosidase activity from adenovirus-infected cells augmented 1.45-fold in the presence of chloroquine and was unaffected by ammonium chloride or monensin (Table 1). This result was at first glance unexpected considering the dogma that adenovirus-mediated disruption of membranes of endocytic vesicles required the acidic pH of endosomes [48,49]. However, validity of this dogma is still a matter of debate. Indeed, recent data indicate that weak base amines do not alter adenovirus uncoating [50] and our results are in agreement with this observation.

The beneficial effect of chloroquine on lipoadenofection efficiency cannot be attributed to the prevention of plasmid DNA degradation by lysosomal nucleases since ammonium chloride and monensin do not augment CAT activity (Table 1). Rather, chloroquine may act by binding DNA in endosomes thereby dissociating lipofectAMINE–DNA complex in a manner similar to that postulated for glycosylated polylysine–DNA complex [51]. Taken together, our results show that endosomotropic agent-linked modulations of gene expression follow a similar pattern after adenoviral infection or lipoadenofection and show opposite traits after lipofection. Hence, delivery of DNA to the cytoplasm during lipoadenofection is likely to be the result of adenovirus endosomolytic activity.

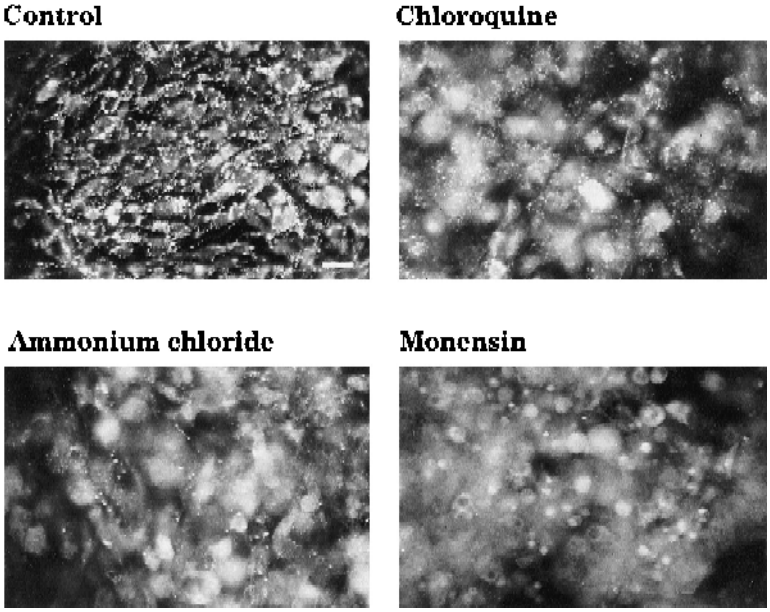

Fig. 5. Acridine orange stained adipocytes. 3T3-F442A adipocytes were incubated in serum-free medium (control: panel A) containing either 100 μM chloroquine (panel B), 10 mM ammonium chloride (panel C) or 5 μM monensin (panel D) for 30 min then 1 μM acridine orange was added for 30 min. Cells were immediately observed under fluorescent microscope with a 546 nm filter. Bar indicates 100 μm.

In conclusion, the association of adenovirus and lipofectAMINE permits the efficient transfer and expression of plasmid DNA in differentiated cells via a process which involves the adenovirus receptor and $\alpha_v\beta_3$ integrin for endocytosis of the ternary complex and in which viral genome is not essential whereas adenoviral endosomolytic activity seems to be required. Determining the structural conformation of the active complex and delineating the mechanism by which adenovirus enhances cationic liposome-mediated gene transfer are prerequisites towards the design of adequate synthetic vectors for gene therapy procedures.

Note added in proof

While this paper was under review, Hong et al. [52] reported that the conserved region of MHC class I $\alpha 2$ domain represents a high affinity receptor for adenovirus type 5 fiber knob.

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