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Cellular uptake of cationic lipid/DNA complexes by cultured myoblasts and myotubes

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Abstract

Several cationic lipids which are highly efficient for delivering genes in vitro do not increase gene delivery in vivo after an intramuscular injection. In order to elucidate the origin of this phenomenon, we have studied the cellular uptake and intracellular fate of cationic lipid/DNA complexes in vitro on myogenic mouse cells (myoblasts and myotubes) of the C2 cell line and of primary cultures. We used a cationic lipid with a spermine head group and its fluorescent analog, and a fluorescent plasmid obtained by nick-translation. In myoblasts, transgene expression was obtained and lipoplexes were internalized in cytoplasmic vesicles. In myotubes, no transgene expression could be detected and we observed an absence of lipoplex internalization. The in vitro uptake of cationic lipid was inversely correlated with the degree of fusion of C2 cell myotubes cultures.

Keywords: Gene transfer; Cationic lipid; DNA; Myoblast; Myotube; Cellular uptake

1. Introduction

Injection of 'naked' plasmid DNA into skeletal muscle results in efficient transgene expression in myofibers [1–4]. This is in contrast to what is observed in many other tissues, where naked DNA is poorly efficient. Skeletal muscle may thus be one type of tissue able to take up and express naked plasmid DNA. This suggests that a unique cytoarchitectural feature of striated muscle is responsible for this phenomenon. T-tubules and caveolae might play a role in the uptake of plasmid DNA [5]. Triadin, a protein located in the sarcoplasmic reticulum, might also be involved in this internalization mechanism [6,7].

Several non-viral vectors are used for increasing transgene expression. Among them, cationic lipids have the following advantages: they protect and deliver DNA efficiently, both in vitro and in vivo after intravenous, intracerebral, or intratumoral injection [8]. They are non-immunogenic and are easy to produce in large quantities. Formulation of polyanionic DNA with cationic lipids results in condensed particles, referred to as lipoplexes [9,10]. It seems clear that cationic lipids induce the cellular uptake of the lipoplexes by condensing the DNA and generating a

Abbreviations: BSA, bovine serum albumin; CMV, cytomegalovirus; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HS, horse serum; Luc, luciferase; PBS, phosphate-buffered saline, pH 7.4; X-gal, 5-bromochloro-3-indolyl-β-D-galactopyranoside

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particle with specific size and positive charge surface. Because of the net negative charge exhibited by the plasma membrane, lipoplexes probably bind to the cell membrane through ionic interactions. The route and mechanism whereby exogenous DNA enters the cell and reaches the nuclear compartment following internalization remain poorly understood. Most authors suggest that intracellular delivery of DNA occurs via endocytosis [11–13], whereas others propose that fusion events at the plasma membrane are involved in the uptake of DNA [14,15]. Phagocytosis and pinocytosis have also been proposed to account for the internalization process of lipoplexes [16].

Surprisingly, several cationic lipids were found unable to increase gene transfer in skeletal muscle in vivo ([5]; B. Pitard et al., unpublished data). However, gene transfer into myogenic cells in culture needs the use of a vector such as a cationic lipid [5,17].

In the present work, we have studied the cellular uptake and intracellular fate of DNA/cationic lipids complexes in vitro on C2 myogenic cells (myoblasts and myotubes) and on primary muscular culture, using a fluorescent cationic lipid with a spermine head group and a fluorescent plasmid. The in vitro uptake of cationic lipid was inversely correlated with the degree of fusion of C2 cell myotubes cultures.

2. Materials and methods

2.1. Cell culture

C2C12 and C2C7 are subclones of the C2 cell line isolated from adult mice skeletal muscle [18]. Myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), glutamine (2 mM, Gibco) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively, Gibco). Cultures were maintained at 37°C in a 5% CO_2/air incubator. For myotubes cultures, the medium overlying confluent myoblasts cultures was replaced with DMEM containing 0.5% (v/v) FBS, glutamine (2 mM) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively), to induce fusion.

Primary myoblasts cultures were obtained by dissociation with collagenase type I (2 mg/ml, Gibco) and trypsin (0.125%, Gibco) of limb muscle from neonatal normal OF1 mice. Myoblasts were seeded onto 35-mm-diameter gelatin-coated dishes as described [19]. Myoblasts cultures were grown in DMEM supplemented with 10% (v/v) horse serum (HS, Gibco), 10% (v/v) FBS, 1.25% (v/v) chick embryo extract (Gibco), glutamine (2 mM) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Four days later, medium was changed to differentiation medium, i.e. DMEM supplemented with 10% HS, 2 mM glutamine and penicillin/streptomycin.

2.2. Plasmids

pCMV-Luc plasmid (pXL 2621) (D. Debono, Rhône-Poulenc Rorer) carries a cassette containing the enhancer–promoter from the immediate early gene of cytomegalovirus (CMV), the gene coding for the luciferase and the polyadenylation site of SV40.

pCMV-nls-LacZ plasmid (pXL 2997) (S. Somarriba, Rhône-Poulenc Rorer) carries a cassette containing the enhancer–promoter from the immediate early gene of cytomegalovirus (CMV), the gene coding for the β-galactosidase with a nuclear localization signal and the polyadenylation site of SV40.

Plasmids were grown using standard techniques and were purified using Wizard Megaprep kit (Promega, Madison, WI). Fluorescent plasmid was prepared by nick translation with dUTP-Red (Amersham, Les Ulis, France) as described previously [20]. These conditions were optimized to obtain only labeled full-length plasmid DNA, which was checked by gel electrophoresis.

2.3. Cationic lipids preparation and lipofection

Myoblasts from C2 cell lines were seeded 3 days before the experiment into 12-well culture plates (10 000 cells per well). Myotubes from C2 cell lines were obtained from myoblasts culture seeded into 12-well culture plates coated with gelatin (150 000 cells per well) and grown 3 days in 10% FBS, then 3–4 days in differentiation medium.

The lipopolyamine used in this study, RPR 120535 was synthesized and characterized as described previously [21]. Cationic lipid was diluted in 150 mM

NaCl. Plasmid pXL 2621 was diluted in 150 mM NaCl, and mixed with an equal volume of cationic lipid solution. The final DNA/cationic lipid mixture was vortexed, left for 10 min at room temperature, diluted in culture medium in the absence of FBS and then added to the cells that had been previously washed twice with fresh medium without FBS. The lipofection was performed in the following conditions: 1 μg of DNA and 3 nmol of RPR 120535 per culture well. After 2 h at 37°C, 10% (v/v) of FBS or 0.5% (v/v) of FBS was added to the culture wells for C2 myoblasts and C2 myotubes, respectively. The cells were then incubated for 24 h at 37°C in the presence of 5% CO_2. The transfected cells were washed twice with PBS and lysed with 200 μl of cell culture lysis reagent (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100, Promega, Madison, WI, USA). Luciferase expression was quantified on 5 μl of centrifuged lysate supernatant, using a luciferase assay kit (Promega). Light emission was measured by integration over 10 s at 25°C using a Lumat LB9501 luminometer (EG&G, Berthold, Evry, France). Relative light units (RLU) were calculated versus background activity. Light emission was normalized to the protein concentration of each sample, determined using the Pierce BCA assay (Pierce, Rockford, IL, USA).

2.4. Cationic lipid quantification

RPR 121653 is a derivative of RPR 120535, which bears a covalently linked rhodamine moiety [20]. Cells were transfected with 1 μg of DNA, 3 nmol of RPR 120535 and 1 nmol of RPR 121653 per culture well, as described above. The transfected cells were washed twice with PBS, and lysed with 200 μl of cell culture lysis reagent (Promega). The lysate was centrifuged at $12\,000\times g$ for 5 min, and a 100-μl aliquot of the supernatant was used to measure the fluorescence (excitation 544 nm, emission at 590 nm) in a Titertek Fluoroskan II. The amount of cationic lipid RPR 121653 contained in the lysate was determined by comparison with the fluorescent signal obtained with serial dilutions of known amounts of the cationic lipid in the same buffer, and was normalized to the protein concentration of each sample. Statistical analysis was performed with Student's *t*-test.

2.5. Plasmid quantification

The cells were transfected with fluorescent labeled plasmid (1 μg per well) and 3 nmol of RPR 120535, as described above. At the end of the transfection, the cells were washed twice with PBS and trypsinized. The cellular suspensions from eight 4-cm^2 culture wells were pooled and centrifuged at $14\,000\times g$ for 4 min at room temperature. The cell pellet was lysed with 200 μl of cell culture lysis reagent (Promega). The lysate was centrifuged at $12\,000\times g$ for 5 min, and a 100-μl aliquot of the supernatant was used to measure fluorescence (excitation 544 nm, emission at 590 nm) in a Titertek Fluoroskan II. The amount of fluorescent labeled plasmid contained in the lysate was determined by comparison with the fluorescent signal obtained with serial dilutions of known amounts of dUTP-Red, and was normalized to the protein concentration of each sample. Statistical analysis was performed with Student's *t*-test.

2.6. Uptake studies

Myoblasts and myotubes from C2 cell lines were seeded as described above. Primary myotubes were obtained after 7 days in differentiation medium.

Cells were transfected with fluorescent cationic lipid and fluorescent DNA, in the following conditions: 1 μg of DNA, 3 nmol of RPR 120535 and 0.1 nmol of RPR 121653, or incubated with FluoroSpheres (100- or 200-nm-diameter carboxylate-modified microspheres, red fluorescent, at a final concentration of 3.10^9 particles/ml, Molecular Probes, Interchim, Montluçon, France). After 24 h at 37°C, cells were rinsed three times with PBS, then fixed for 10 min in methanol at room temperature and washed with PBS. Nuclei were stained with DAPI (500 ng/ml) for 20 min and the cells were mounted in Mowiol for examination.

Cells were incubated with 1 mg/ml of rhodamine B-dextran conjugates (70 000 MW, Molecular Probes) in DMEM, for 2 h at 37°C, then incubated for 1 h in DMEM, fixed for 10 min in methanol at

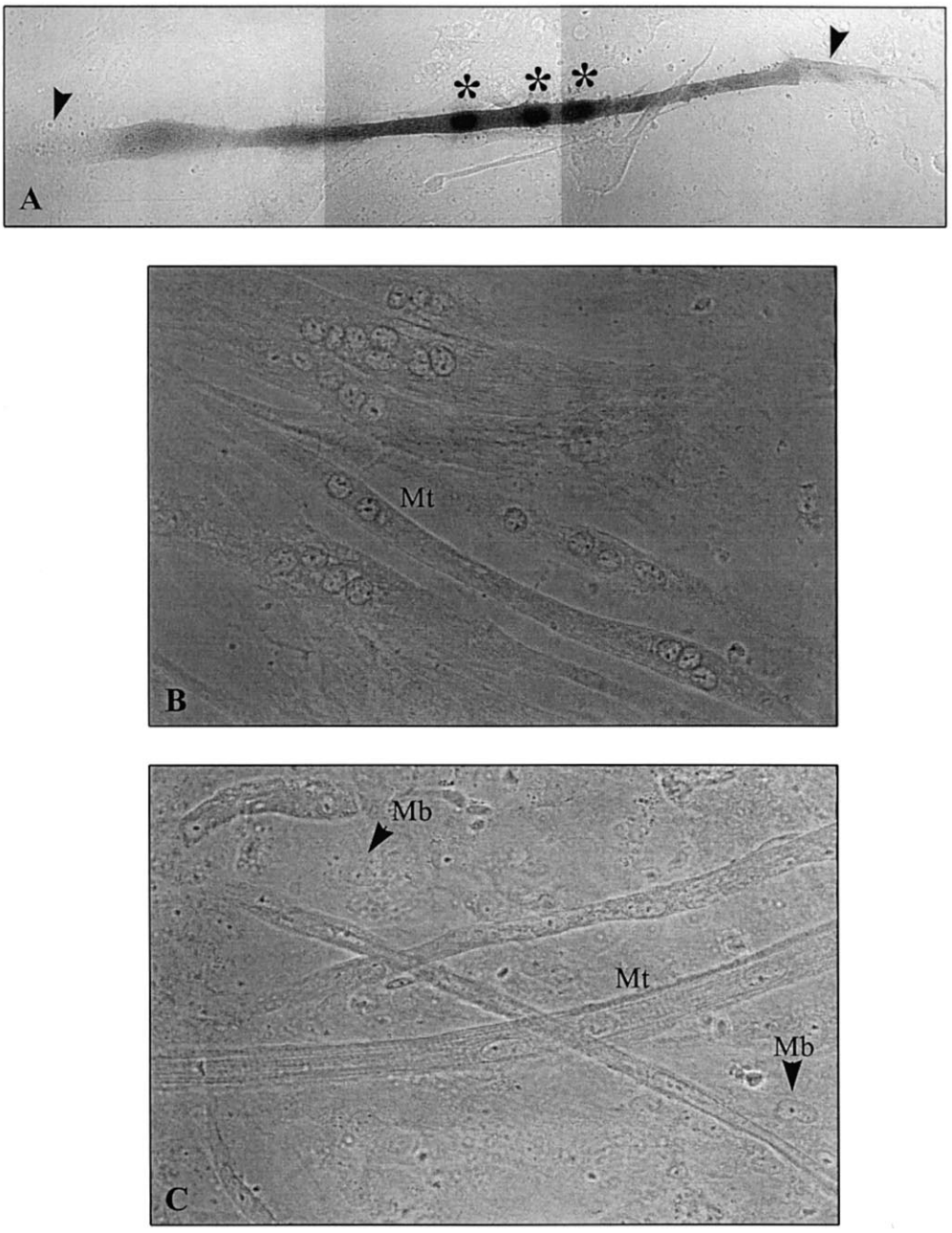


Fig. 1. (A) Expression of nls-β-galactosidase in a myotube from primary culture after nuclear microinjection. One nucleus was injected with pCMV-nls-LacZ plasmid at 0.5 μg/μl and incubated for 4 h at 37°C. nls-β-galactosidase was revealed with X-gal staining (see Section 2). Note the presence of a diffuse staining around nuclei expressing nls-β-galactosidase (marked with a star). Arrows indicate the nuclei of the same myotube where no expression of the transgene was detected. (B) Phase contrast of C2C12 cell line and (C) phase contrast of primary muscular culture. Mt indicates myotubes and arrows show myoblasts (Mb) present in the differentiated culture. Note the presence of cross-striation in one primary cultured myotube.

room temperature, stained with DAPI and mounted for observation.

Slides were observed with a Zeiss Axiophot microscope equipped with phase-contrast and epifluorescence optics (Zeiss, Le Pecq, France). Images were captured with a cooled CCD camera (Hamamatsu) and analyzed with Samba imaging software (Unilog, Meylan, France).

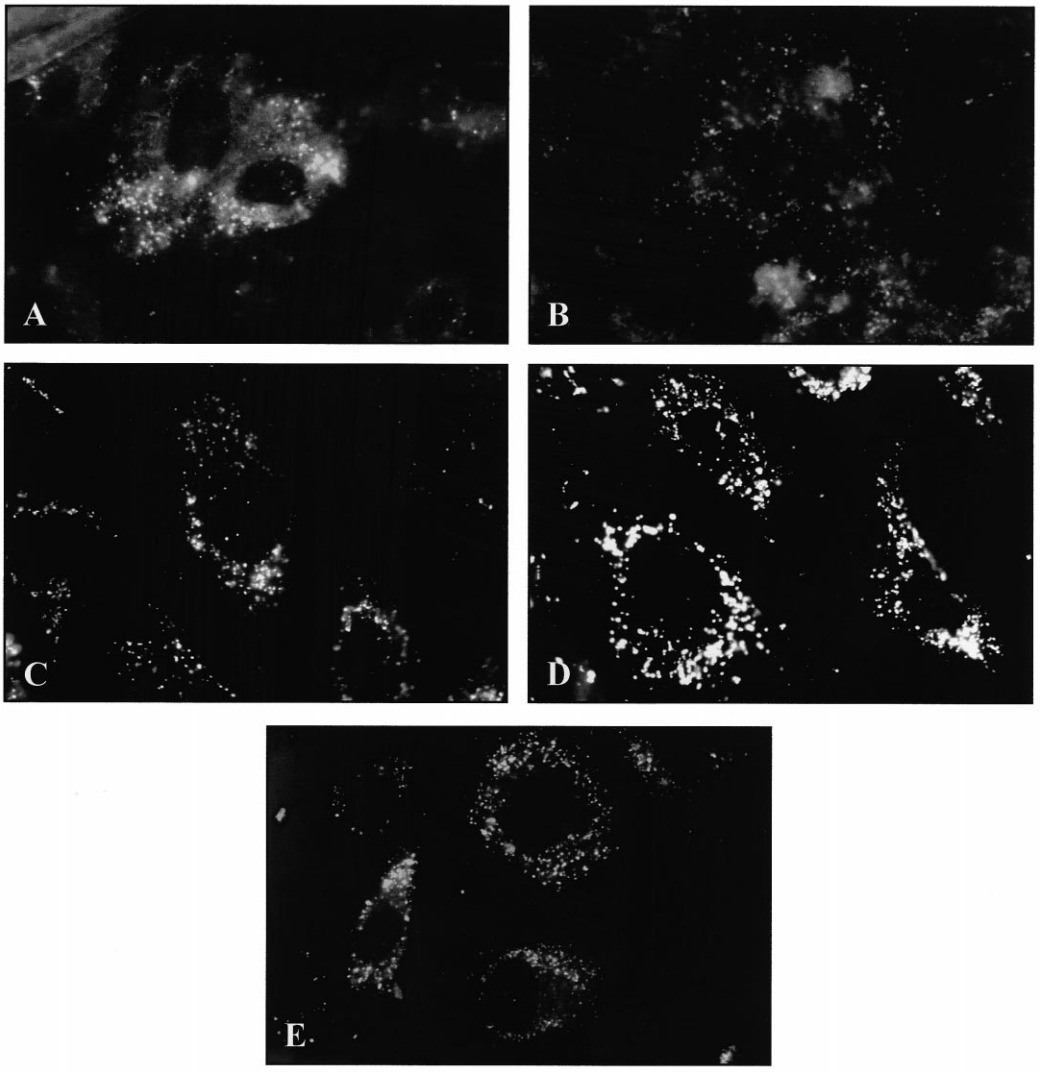


Fig. 2. Fluorescence microscopy of myoblasts from primary culture incubated with fluorescent cationic lipid, fluorescent DNA, fluorescent dextran, or FluoroSpheres. The cells were treated as indicated in Section 2. Myoblasts were transfected with labeled cationic lipid (3 nmol RPR 120535 and 0.1 nmol RPR 121653 per μg of plasmid) (A), or with labeled DNA (1 μg DNA with 3 nmol RPR 120535) (B), and examined after 24 h. Myoblasts were exposed to 100 nm (C) or 200 nm (D) FluoroSpheres for 24 h at 37°C. (E) Myoblasts were exposed for 2 h at 37°C to rhodamine dextran (1 mg/ml in DMEM).

2.7. *Immunofluorescence staining for desmin*

After fixation, cells were washed and incubated for 30 min in blocking solution (PBS containing 0.5% BSA and 0.05% saponin (w/v)), followed by incubation for 30 min with monoclonal anti-desmin antibody (1/100, clone DE-U-10, Sigma-Aldrich, Saint Quentin Fallavier, France) followed by FITC conjugate sheep anti-mouse IgG (1/100, Sigma-Aldrich). Nuclei were stained with DAPI (500 ng/ml) for 20 min and the cells were mounted in Mowiol for examination.

2.8. *Microinjection and β-galactosidase histochemistry*

The solution of DNA (pCMV-nls-LacZ) was injected into one myotube nucleus via glass micropipettes having tip diameters ranging from 0.3 to 0.7 μm (Femtotips, Eppendorf, Hamburg, Germany) at

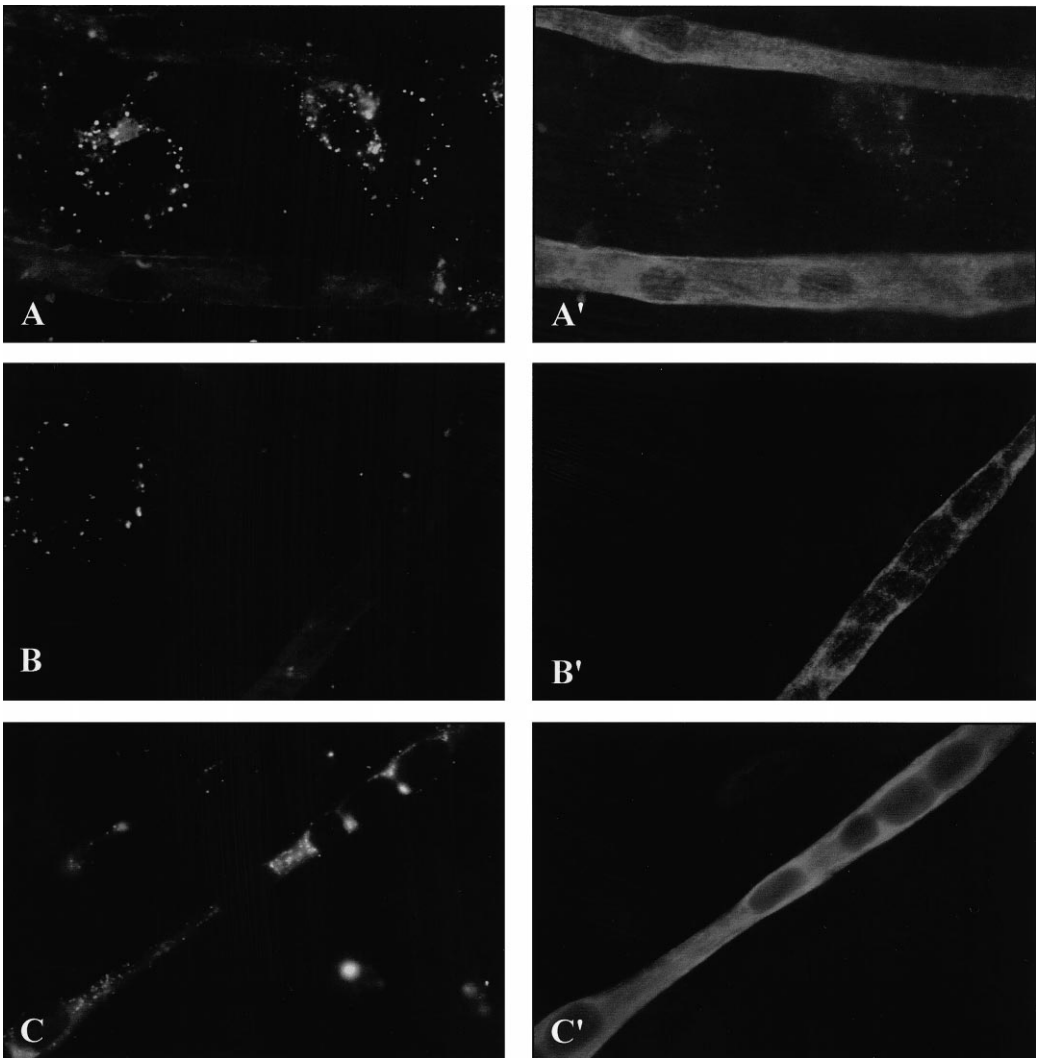


Fig. 3. (A–F) Fluorescence microscopy of myotubes from primary culture transfected with fluorescent cationic lipid, fluorescent DNA or incubated with 70 kDa rhodamine dextran or FluoroSpheres. (A′–F′) Same field as A–F, but myotubes were labeled by immunocytochemistry with anti-desmin antibodies and FITC-conjugated secondary antibodies. Myotubes were transfected with labeled cationic lipid (3 nmol RPR 120535 and 0.1 nmol RPR 121653 per μg of plasmid) (A,A′), or with labeled DNA (1 μg DNA with 3 nmol RPR 120535) (B,B′). Myotubes were exposed for 2 h at 37°C to rhodamine dextran (C,C′) (1 mg/ml in DMEM). Myotubes were exposed to FluoroSpheres with 100 nm (D,D′ and E,E′) or 200 nm diameter (F,F′).

0.5 μg/μl concentration. Injections were carried out under visual control on a fixed stage of an inverted phase contrast microscope (Axiovert 135, Zeiss) using a Micromanipulator 5171 and a Microinjector 5242 (Eppendorf). The average volume injected into each nucleus (0.1–0.2 pl) was determined by injecting ^{35}S-dATPαS into cells and measuring the radioactive content [22]. This corresponded to average 6000–12 000 plasmid molecules per nucleus. Following injection, the cells were incubated for 4 h at 37°C in a 5% CO_2 incubator to allow for reporter gene expression. Cells were washed three times with PBS before fixation in 1% formaldehyde and 0.2% glutaraldehyde for 10 min at room temperature. Further permeabilization and three washes were performed in PBS containing 2 mM $MgCl_2$, 1 mM EGTA and 0.02% Nonidet P-40. Finally, samples were incubated for 2 h at 37°C in the chromogenic substrate X-Gal in permeabilization solution containing 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$ and

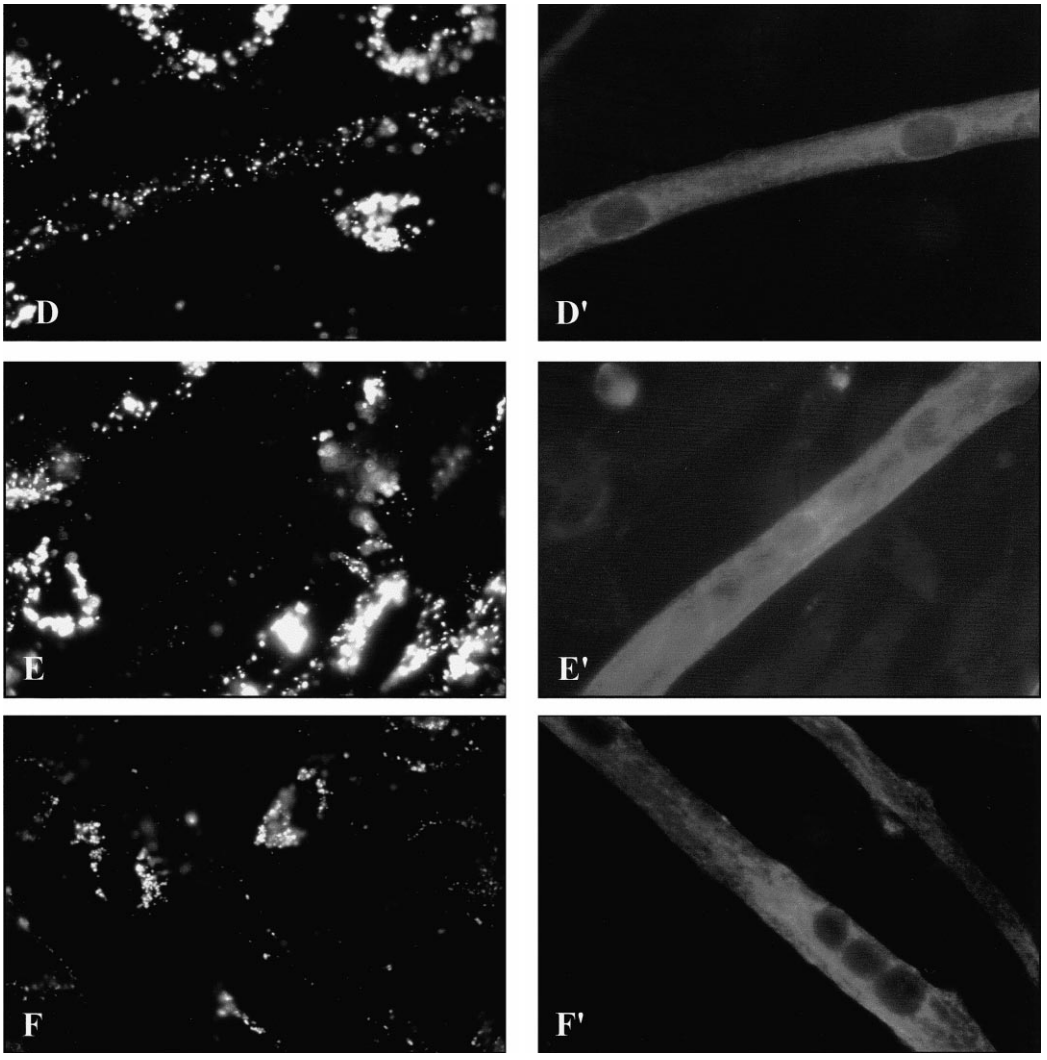


Fig. 3 (*continued*).

0.4 mg/ml 5-bromochloro-3-indolyl-β-D-galactopyranoside (X-Gal). Cells were mounted in Mowiol for examination.

3. Results

3.1. Gene transfer efficiency

As a cationic lipid model, we used RPR 120535, a spermine derivative which was shown to transfect many cell types in vitro and in vivo [21]. The myogenic cells were transfected for 2 h with RPR 120535/pCMV-Luc complexes (3 nmol lipid/μg DNA) in the absence of serum, followed by 24 h of incubation in the presence of serum. As shown in Table 1, the luciferase activity was not significantly different between the two myogenic cell lines (C2C12 and C2C7), but was about 10 000-fold higher in myoblast than myotube cultures, in both cell lines. We asked whether this decrease in lipofection efficiency: (1) was a consequence of the incapacity of myotubes to transcript and translate an exogenous transgene; or (2) was due to a difference in the uptake of lipoplexes between myoblasts and myotubes.

The first hypothesis was studied by nuclear microinjection of nls-β-galactosidase encoding plasmids in myotubes. As shown in Fig. 1A, myotubes could express the microinjected transgene. Note that the nls-β-galactosidase protein was able to diffuse in

the whole myotube, since only one nucleus was injected per cell. Thus, differentiated myotubes in vitro can readily express a transgene.

3.2. Uptake of lipoplexes

We quantified the amount of plasmid DNA and cationic lipid recovered in transfected cells 24 h after lipofection. We took advantage of a rhodamine-labeled cationic lipid analog to RPR 120535, referred to as RPR 121653, to assay the amount of cationic lipid associated with, or taken up by myogenic cells. As indicated in Table 1, for the C2C12 cell line, there was no significant difference between the amount of cationic lipid taken up by myoblasts and myotubes, and for the C2C7 cell line, the amount of RPR 121653 internalized was 2-fold higher in myoblasts than in myotubes. To examine if these results could be influenced by the presence of labeled lipid that was simply bound to plasma membrane but not endocytosed, we performed two series of controls. First, we quantified labeled lipid in C2C12 cells harvested immediately following contact with the lipoplexes. In these conditions, less than 0.04 pmol of RPR 121653 per μg of protein were detected associated with the cells. Second, we compared the amount of lipid associated with the cells after incubation with the lipoplexes for 2 h at 37 and 4°C, where endocytosis is inhibited. We found that the uptake of lipid was reduced by 60% at 4°C. This suggests that around 40% of the lipid associated to the cells is due to simple binding of the lipoplexes to the plasma membrane.

We also used rhodamine-labeled plasmid to estimate the amount of internalized DNA. As shown in Table 1, the amount of DNA internalized was not significantly different between the two myogenic cell lines (C2C12 and C2C7), but was about 3-fold higher in myoblasts than in myotube cultures, in both cell lines.

It is important to note that in all the myotube cultures, residual proliferating myoblastic cells were always observed (Fig. 1B,C). Consequently, the global quantification of luciferase activity, of cationic lipid and of plasmid uptake in differentiated cultures could not discriminate between subpopulations of myoblasts and myotubes. In myotubes cultures transfected with β-galactosidase encoding plasmids, the expression was exclusively detected in myoblasts (data not shown).

3.3. Effect of myogenic differentiation on lipoplex intracellular localization

We next investigated whether myogenic differentiation had an effect on the intracellular localization of the lipoplexes. The intracellular localization of the lipoplexes was analyzed by fluorescent microscopy, using either the fluorescent cationic lipid RPR 121653 or a nick-translated plasmid with fluorescent dUTP-red. Rhodamine-dextran, or rhodamine-labeled carboxylate-modified microspheres with known diameter (FluoroSpheres) were also used as controls. Fluorescent microscopy makes it possible to precisely analyze the internalization of cationic lipid/DNA complexes in cultures containing both myoblasts and myotubes.

We found that cationic lipid and plasmid DNA were present in 100% of mononucleated myoblasts from primary culture, displaying a punctated cytoplasmic staining, mostly in the perinuclear region (Fig. 2A,B). Similarly, myoblasts were shown to endocytose 100- or 200-nm microspheres (Fig. 2C,D) and fluorescent dextran (Fig. 2E). Similar results

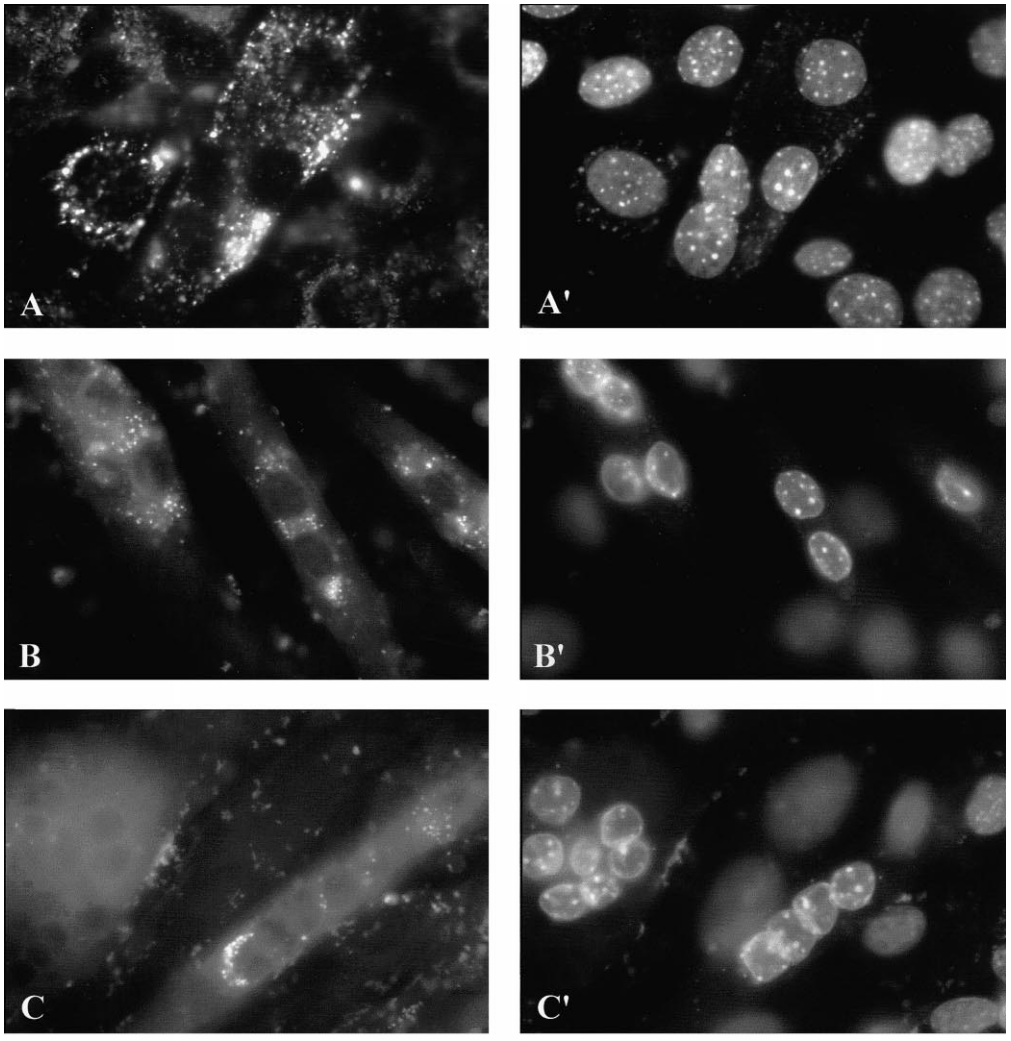
Table 1
Comparison of cationic lipid-mediated gene transfer, cationic lipid uptake (RPR 121653) and DNA uptake by myoblasts and myotubes from C2C12 and C2C7 cell lines

	C2C12		C2C7	
	Myoblasts	Myotubes	Myoblasts	Myotubes
Transfection (RLU/μg protein)	$5.95 \pm 2.49 \times 10^5$ (12)	88 ± 76 (12)	$9.72 \pm 1.80 \times 10^5$ (6)	62 ± 71 (12)
RPR 121653 uptake (pmol/μg protein)	1.97 ± 0.79 (12)[a]	1.55 ± 0.05 (12)[a]	2.38 ± 0.86 (12)[c]	1.14 ± 0.66 (12)[c]
Fluorescent DNA uptake (ng/μg protein)	0.97 ± 0.21 (3)[b]	0.33 ± 0.21 (3)[b]	0.75 ± 0.17 (6)[c]	0.20 ± 0.04 (6)[c]

Statistical difference between myoblasts and myotubes for each cell line (number of wells): [a]not significant; [b]$P < 0.05$; [c]$P < 0.001$.

were obtained with myoblasts of the C2C12 and C2C7 cell lines (data not shown).

On the contrary, no vesicular staining was observed in myotubes from primary muscular cultures, whereas a clear uptake of fluorescent lipoplexes was detected in myoblasts from the same well (Fig. 3A,B). Rhodamine-labeled dextran was endocytosed by myotubes (Fig. 3C). The size of FluoroSpheres clearly influenced their internalization by myotubes. Some myotubes were able to take up microspheres



Conditions	A-A'	B-B'	C-C'
% total myotubes	<1 %	22.8 %	33.7 %
% myotubes with lipoplexes	100 %	74.1 %	51.3 %

Fig. 4. Fluorescence microscopy of C2C12 myotubes transfected with fluorescent cationic lipid (3 nmol RPR 120535 and 1 nmol RPR 121653 per μg of plasmid) (A–C). Nuclei were stained with DAPI (A′–C′). The percentage of myotubes was calculated as the number of nuclei residing within any cell containing more than two nuclei divided by the total nuclear count in a field of view. Five separate fields were scored for each differentiation state. Note that cationic lipid uptake by myotubes was inversely related with the degree of fusion.

with 100 nm diameter (Fig. 3D), whereas other myotubes did not (Fig. 3E). Microspheres with 200 nm diameter were never detected in differentiated cells (Fig. 3F).

Using the C2C12 cell line, we studied the uptake of fluorescent lipoplexes as a function of cell fusion, which is an estimate of myotubes differentiation. Cultures incubated in differentiation medium for various periods of time and showing different levels of fusion were examined. The percentage of myotubes in C2C12 cultures was taken as the number of nuclei residing within any cell containing more than two nuclei, divided by the total nuclear count in a view field [23]. We found that the uptake of cationic lipid by myotubes was inversely related to the degree of fusion (Fig. 4).

4. Discussion

In the present work, we show that the efficiency of transfection of pCMV-Luc plasmid complexed with cationic lipids was dependent upon the differentiation state of the muscle cell cultures in vitro, with decreasing expression of a reporter gene as myoblasts fuse into myotubes. These results are in agreement with earlier reports obtained with different lipid formulations, in which it was suggested that cell division might be critical for efficient gene transfer [17,24]. Indeed, mitosis might be requisite for exogenous DNA to enter the nucleus and to access the nuclear transcriptional machinery, because during cell proliferation the nuclear envelope is transiently dispersed. Although this hypothesis might be valid, our present studies with fluorescent cationic lipid and fluorescent plasmid suggest that the absence of transgene expression in myotubes cultures can be explained by a low efficiency of lipoplex internalization. Quantification of the amount of fluorescent plasmid recovered in transfected cells after lipofection indeed showed that the uptake of DNA was 3-fold higher in myoblasts cultures than in differentiated cultures enriched in myotubes. The amount of cationic lipid associated with the cells was not different between myoblasts and myotubes of the C2C12 cell line and was 2-fold higher in myoblasts than in myotubes of the C2C7 cell line. These results can be explained by the fact that a significant part of the fluorescence associated with the cells was due to lipid bound to the plasma membrane and not internalized by endocytosis. Moreover, these assays cannot distinguish between lipoplexes associated with myotubes and those associated with myoblasts that are always present in the culture wells.

Consequently, we followed the cellular internalization of fluorescent lipoplexes by microscopy. Our results show an absence of lipoplex endocytosis by myotubes, whereas myoblasts take up a large amount of lipoplexes recovered inside cytoplasmic subcellular compartments. Pictures obtained with RPR 121653 generally showed a non-specific diffusion of this fluorescent cationic lipid on the whole myotube surface (Figs. 3 and 4), again suggesting that a significant part of RPR 121653 was bound to the plasma membrane. Incubation with dextran showed that there was no difference on fluid phase endocytosis between myoblasts and myotubes-enriched culture (Fig. 3C). On the other side, using size-calibrating fluorescent latex particles (FluoroSpheres), we determined that myotubes could not take up particles greater than 200 nm in diameter. Recently, it was determined that our cationic lipid/DNA complexes preformed in 150 mM NaCl, form stable particles in the 500–1000-nm range in DMEM without serum [20]. This suggests that lipoplexes formed with RPR 121653 cannot be internalized by myotubes because of their size. On the contrary, mononucleated cells, such as fibroblasts [20] or myoblasts (this study), have the capacity to take up large particles, with a diameter up to 500 nm.

The relevance of these results to the in vivo situation has to be further analyzed, since non-coated DNA was not taken up by cultured myotubes, in contrast to the in vivo gene transfer data.

The differentiation of skeletal myogenic cells is characterized by: (1) myoblasts fusion; (2) synthesis of myogenic factors; (3) cell division arrest [25]; (4) synthesis of extracellular components that facilitate adhesion to the tissue support [26]; and (5) formation of specific cellular structures, such as sarcomeres, sarcoplasmic reticulum [27], and transverse tubules [28]. Any of these factors could be responsible for the differences in particle internalization by myogenic cells during their cellular differentiation. A change of the surface charge might also influence the entry of cationic lipid/DNA complexes into myogen-

ic cells, as observed in differentiated airway epithelial cells [16].

In conclusion, the differentiation state of myogenic cells influences their capacity to internalize DNA from plasmid/cationic lipid complexes in vitro. In myoblasts, high transgene expression and lipoplex (RPR 121653/DNA complexes) internalization was observed, as in fibroblasts [20], whereas, in cultured differentiated myotubes, no transgene expression was detected and this absence of expression was correlated with the absence of lipoplex internalization.

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