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Cationic liposomes improve stability and intracellular delivery of antisense oligonucleotides into CaSki cells

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Abstract

Antisense oligonucleotides (ODNs) are promising novel therapeutic agents against viral infections and cancer. However, problems with their inefficient delivery and inadequate stability have to be solved before they can be used in therapy. To circumvent these obstacles, a wide variety of improvements, including phosphorothioate ODNs and liposomes as a carrier system, have been developed. This study was designed to compare the effects of two cationic liposomes on the intracellular delivery and stability of ODNs in CaSki cell cultures. Also the stability of 3′-end phosphorothioate ODNs were investigated. The 3′-modification neither had any effect on the delivery, nor protected the ODNs against degradation. The cellular delivery and stability of ODNs was improved with both cationic liposomes, but a cationic liposomal preparations containing dimethyldioctadecylammonium bromide and dioleoylphosphatidylethanolamine (DDAB/DOPE) was more efficient than commercially available *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammoniummethylsulfate (DOTAP). The improved cellular delivery was largely due to the stabilization of ODNs by cationic liposomes. The improved stability in the culture medium indicates that the cationic liposomes per se protect the ODNs from enzymatic degradation. Indeed, intact ODNs were found in the cytoplasm and nucleus only when delivered by cationic liposomes.

Keywords: Antisense oligonucleotide; Cationic liposome; CaSki cell

1. Introduction

Antisense oligonucleotides (ODNs) designed to hybridize to specific mRNA or double-stranded DNA sequences are potential therapeutic agents against viral infections and cancer [1–4]. Before practical applications of ODNs a number of problems, including poor biological stability and cell delivery have to be solved. Therefore, modified analogs of ODNs have been developed, e.g., the phosphorothioate ODNs [3–5]. Although the fully thioated (and other modified) ODNs are more resistant to nucleases, they have several non-sequence-specific effects, including reduced hybridization, non-selective protein binding, alteration in cell morphology and proliferation [6–10]. To reduce these side-effects partially modified phosphodiester ODNs have been developed [11]. It was recently shown that even a single 3′-phosphorothioate linkage increased the stability of ODNs in the culture medium [12,13]. Also some recent modifications of ODNs, like cholesterol and poly(L-lysine) conjugations, seem to improve their cellular uptake [14,15].

The mechanisms by which antisense ODNs enter the cells are not fully understood. Some evidence is available on the existence of ODN-receptors on the cell surface [16,17], and consequently, the uptake of ODNs has been shown to depend on the time and concentration [6,17–19]. Their cellular stability and uptake without chemical modifications or carriers is ineffective [20]. Phosphatidylcholine liposomes have proved to be efficient carriers of ODNs. At the same time, these liposomes increase the stability of ODNs in culture medium [18,21,22]. Of the liposomes,

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especially cationic liposomes can deliver ODNs efficiently into the cells [23–28] due to complexing the anionic ODNs on their surface on the basis of electrostatic interactions [22]. Most of the studies have dealt with *N*-(1-(2,3-dioleyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) [23–28]. Recently, a new cationic liposome containing DDAB and DOPE was introduced showing an efficient delivery of DNA into cells [29]. The effects of DDAB/DOPE on the delivery of antisense ODNs has not been studied by other groups.

The aim of the present study was to compare the effects of DDAB/DOPE and commercially available DOTAP on the delivery and stability of ODNs in the CaSki cells. The stability of ODNs was analysed in the culture medium, cytoplasm, and nucleus. CaSki cells (a human cervical carcinoma-derived cell line containing human papillomavirus (HPV) 16 DNA) were used with the future aim to develop a local, liposomal antisense therapy against HPV infections. The effects of sulfurization of the 3′-phosphodiester linkage and the length of ODN on its degradation and cellular uptake were also studied.

2. Materials and methods

2.1. Cell culture

CaSki cells, a human cervical cancer cell line containing HPV 16 DNA (approx. 600 copies), were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated (at 56°C for 60 min) fetal calf serum (Gibco, Paisley, Scotland, UK) at 37°C in 5% CO_2. The experiments were performed on 24-well plates (Nunc, Roskilde, Denmark) with 10^5 cells/well in a 500 μl volume. ODNs and liposomes were added 12 h after seeding of the cells, and incubated for 2, 5 or 8 h. The concentrations of ODNs and liposomal oligonucleotides were 5 and 0.1 μM.

2.2. Antisense oligonucleotides

Antisense (phosphodiester) oligonucleotides and 3′-end modified phosphorothioate analogs (AntiSense reagent, Pharmacia, Uppsala, Sweden) were synthesized on an automated DNA synthesizer (Gene Assembler Plus, Pharmacia, Uppsala, Sweden). These ODNs were complementary to the initiation region of HPV 16 early 7 gene, which is one of the genes coding the major transforming proteins in HPV-induced malignancies [30]. In the present study, following ODNs were used: unmodified 12- and 23-mers and their 3′-end sulfurized analogs. The sequences of unmodified and modified 12-mers and 23-mers were 5′-ATCTCCATGCAT-3′ and 5′-ATCTCCATGCAsT-3′, 5′-AATGTAGGTGTATCTCCATGCAT-3′ and 5′-AATGTAGGTGTATCTCCATGCAsT-3′, respectively.

The ODNs were purified by reverse-phase fast protein liquid chromatography (FPLC system, Pharmacia, Uppsala, Sweden) on a PepRPC HR 5/5 reverse-phase column (Pharmacia, Uppsala, Sweden), and eluted by an acetonitrile gradient (5–30%, BDH), in 0.1 M triethylammonium acetate buffer (Fluka, Buchs, Switzerland). The equivalent molar amounts of 12- and 23-mers were 5′-end labelled with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham, UK) using T4 polynucleotide kinase (Biolabs, Beverly, MA, USA) at 37°C for 45 min as recommended by the supplier and precipitated by ethanol to remove the non-reacted [^{32}P]ATP.

2.3. Liposomes

Two cationic liposomes were used, a commercially available Transfection-reagent DOTAP (Boehringer-Mannheim, Pentzberg, Germany), and a cationic liposomal preparation containing dimethyldioctadecylammonium bromide (DDAB) and dioleoylphosphatidylethanolamine (DOPE) (2:5, w/w) as described previously [29]. The size distribution of the lipid dispersions was determined by quasielastic light scattering (Nicomp Submicron Particle Sizer, Model 370, Santa Barbara, CA). The mean diameters of liposomes were 86 nm (DDAB/DOPE) and 43 nm (DOTAP). In the experiments, the final concentration of DOTAP or DDAB was 10 μM. The concentration of both liposomal ODNs (12- and 23-mers) was 0.1 μM. Thus, the $-/+$ charge ratios (ODN/cationic lipid) were 0.12 and 0.23 giving a net positive charge to ODN-liposome-complexes to allow fusion between the cell membranes and the ODN-liposome complexes. Previously similar charge ratios of oligonucleotides to Lipofectin™ (DOTMA/DOPE, 1:1, w/w) have been used succesfully [25,27].

2.4. Delivery and stability experiments

After incubation with ODNs and liposomes, the culture medium was removed, and the cells were washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.2, containing 1% bovine serum albumin (BSA). After washings, the cells were trypsinated, washed again with PBS containing BSA and centrifuged $1000 \times g$ for 5 min. Separation of the cell membrane-binding fraction was performed by using the acid-salt elution method as described by Haigler et al. and modified by Gao et al. [31,32]. The cell pellet was suspended with a solution of 0.2 M acetic acid and 0.5 M sodium chloride, pH 2.5, at 4°C for 10 min to eluate the surface-bound ligands and centrifuged $1000 \times g$ for 5 min. The cell-associated radioactivity resistant to the acid-salt treatment represented the intracellular ODNs. Delivery of ODNs into the nuclear fraction was determined according to the method of Roop et al. and modified by Gao et al. [32,33]. In brief, the cells were washed with a solution of 0.5% Nonidet P-40, 0.25 M

sucrose and 1.5% citric acid for 10 min at 4°C, and centrifuged $1000 \times g$ for 10 min. The washings were repeated three times. The radioactivity of the supernatants and the final pellet resistant to Nonidet P-40 treatment represented the cytoplasmic and the nuclear fractions, respectively.

The radioactivities of the culture medium, washings, supernatants and pellets were determined by the method of Cerenkov in a liquid scintillation counter (Wallac 1409, Pharmacia, Uppsala, Sweden). The stability of ODNs in the culture medium, cytoplasmic fraction and nuclear fraction were analyzed using 20% polyacrylamide gel (PAGE) in 7 M urea. The samples of ODNs in the medium were analyzed per se, the intracellular ODNs were analyzed after separating the membrane-bound fraction, and the nuclear ODNs after separating both the membrane-bound and the cytoplasmic fractions. ODN sizing markers contained 8 to 32 bases (Pharmacia, Uppsala, Sweden). A 2 μl sample was mixed with 4 μl of loading buffer (96% formamide, 20 mM EDTA, 0.05% Bromophenol blue, and 0.05% xylene cyanol) and applied to the gel. Electrophoresis was performed at 45 W for 3.5–5 h at room temperature. After fixation, the gels were dried at 65°C for 2 h and exposed to Kodak XAR film at −70°C for 12 h to 11 days with an intensifying screen.

2.5. Delivery and stability of non-liposomal oligonucleotides

At first, the delivery and stability of unmodified and 3′-end modified (phosphorothioate) 12- and 23 mers were compared. Approx. 10^6 cpm of labelled and 5 μM of unlabelled ODNs were added into each well, and incubated for 2, 5 and 8 h. After incubation, the delivery and stability of the ODNs were analyzed as described above. Cellular delivery of the ODNs was studied in triplicate in a single experiment. The stability of the ODNs was analyzed twice from the different samples (medium, cytoplasm, nucleus) in separate gel runs.

2.6. Delivery and stability of liposomal oligonucleotides

The delivery and stability of 3′-end modified 23- and 12-mers with cationic liposomes were studied. At the beginning of the experiments, labelled and unlabelled ODNs were mixed, and incubated with DDAB/DOPE or DOTAP liposomes at room temperature for 10 min. Approx. 10^6 cpm of labelled and 0.1 μM of unlabelled 12- or 23-mers with or without liposomes (at concentration of 10 μM of cationic DDAB or DOTAP) were added into each well, and incubated for 2, 5 and 8 h. After incubation, the cellular uptake and stability were analyzed as described above. The delivery experiments of 12- and 23-mers were carried out in triplicates. The stability of 12- and 23-mers was analysed twice from different samples in separate gel runs.

3. Results

3.1. Delivery and stability of non-liposomal oligonucleotides

After incubation with the unmodified and 3′-end modified ODNs, the cell-associated radioactivity was 0.1–3.2% (Fig. 1a). The cell associated radioactivity of all ODNs was approx. 7–13-fold higher after 8 h than after 2 h. Cellular delivery of the 12-mers was slightly more efficient than that of the 23-mers. The modification of ODNs at the 3′-terminus did not have any effect on the cell associated radioactivity. After 8 h, the nucleus-associated radioactivity of all ODNs was under 0.4% (Fig. 1b). At all

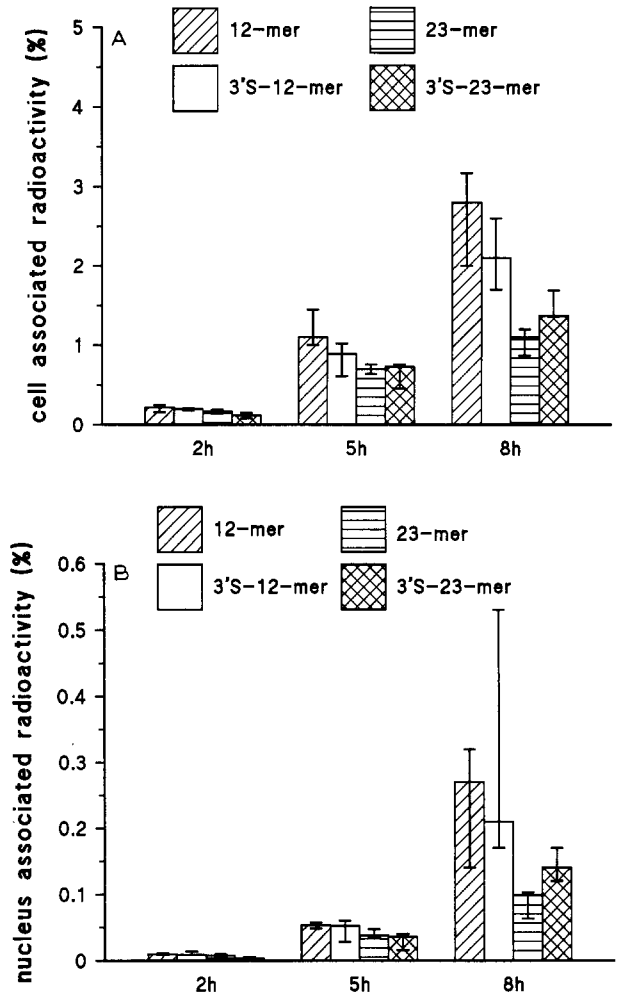


Fig. 1. The percentage of cell-associated radioactivity (a) and nucleus-associated radioactivity (b). The cellular and nuclear uptake of unmodified and 3′-end modified 12- and 23-mers were compared. The CaSki cells were incubated with the ODNs as described in Materials and methods. The concentration of ODNs was 5 μM. After 2, 5 and 8 h of incubation, the different fractions were extracted from the cells and measured by liquid scintillation counter. The radioactivity associated with the cells (a, sum of the counts from the cell membrane, cytoplasmic and nuclear fractions) and nucleus (b) is presented as percentages of the total counts added to the well. The experiment was carried out as triplicates, and the error bars represent the range of the data.

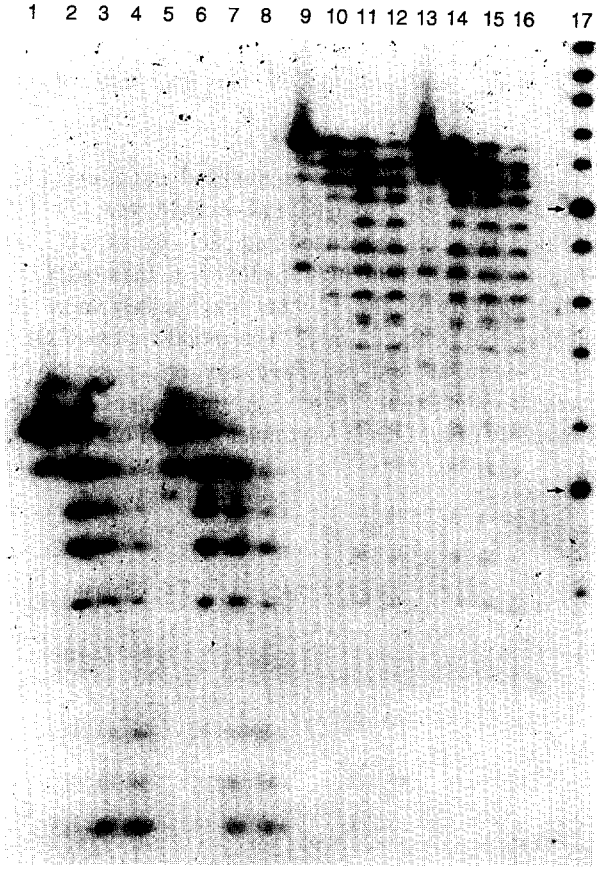


Fig. 2. The stability of 12- and 23-mers in the culture medium. The unmodified and 3'-end modified 12- and 23-mers were incubated in CaSki cell cultures. The concentration of ODNs was 5 μM. After incubations, the samples from the culture medium were analysed by 20% PAGE in 7 M urea. Lanes 1–4: 12-mer after 0, 2, 5, and 8 h of incubation. Lanes 5–8: modified 12-mer after 0, 2, 5, and 8 h of incubation. Lanes 9–12: 23-mer after 0, 2, 5, and 8 h of incubation. Lanes 13–16: modified 23-mer after 0, 2, 5, and 8 h of incubation. Lane 17: ODN- size markers: range 8–30 bases. 10- and 20-mers are marked by arrows.

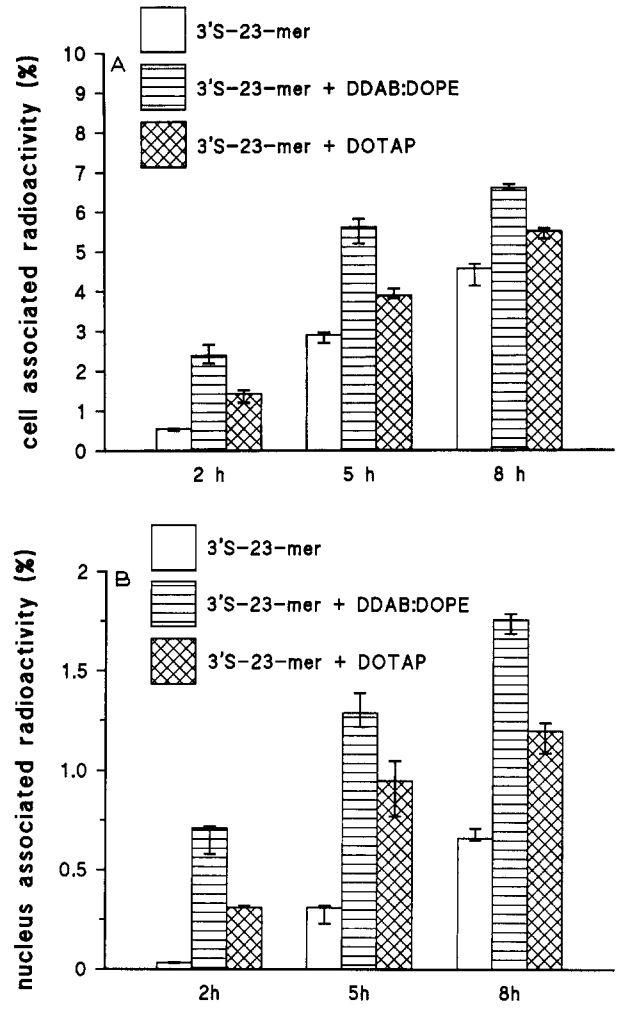


Fig. 3. The percentage of cell-associated radioactivity (a) and nucleus-associated radioactivity (b) after ODN delivery with the cationic liposomes. The 3'-end modified 23-mer ODNs were incubated with the DDAB/DOPE or DOTAP. The concentration of ODNs was 0.1 μM, and cationic liposomes 10 μM. After incubations, the cellular fractions were extracted and analysed as described in Fig. 1. The cell- and nucleus-associated radioactivities were presented as percentages of the total counts added into the well. The experiments were carried out as triplicates, and error bars represent the range of the data.

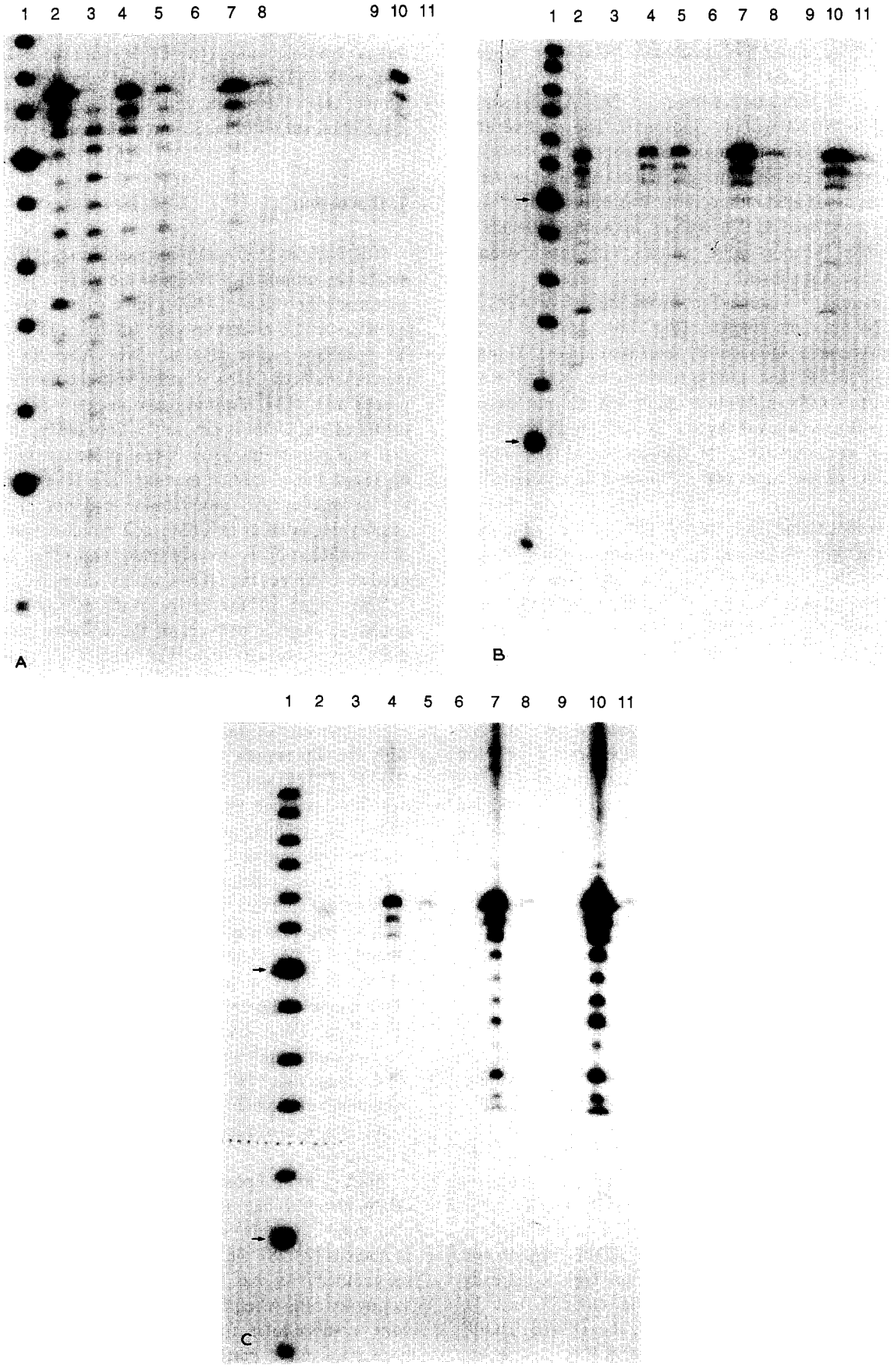
times, approx. 40% of the cell-associated radioactivity localized in the plasma membrane with all ODNs (variation 35–55%). After 2 h, approx. 55% of the cell-associated radioactivity was found in the cytoplasm and 5% in the nucleus, while after 8 h, the radioactivity decreased to 50% in the cytoplasm and increased to 10% in the nucleus.

Fig. 2 shows the degradation of ODNs in the culture medium. The modification of ODNs at the 3'-end with a thioate group did not increase their stability. Already after 2 h of incubation, part of the 23-mers (lanes 10 and 14) and most of the 12-mers (lanes 2 and 6) were degraded, and after 8 h, nearly all the 23-mers were degraded (lanes 12 and 16) and no intact 12-mers were seen (lanes 4 and 8). The repeated PAGE showed similar results.

3.2. Delivery and stability of liposomal oligonucleotides

Both cationic liposomes, DDAB/DOPE and DOTAP, improved the cellular delivery of the 3'-end modified

Fig. 4. The stability of modified 23-mers with the liposomes in the culture medium (a), cytoplasm (b), and nucleus (c). The 3'-end modified 23-mers were incubated with the cationic liposomes. The concentration of ODNs and cationic liposomes were 0.1 and 10 μM, respectively. After incubations, the samples from the culture medium, cytoplasm and nucleus were analyzed by 20% PAGE in 7 M urea. The concentrations of the control ODNs were 0.1 (a), 0.005 (b), and 0.001 (c) μM, respectively. Lane 1: ODN-size markers, range 8–26 (a and b) and 8–32 (c) bases. 10- and 20-mers are marked by arrows. Lane 2: control ODN, modified 23-mer without incubation. Lanes 3–5: modified 23-mer without liposomes, with DDAB/DOPE, and with DOTAP after 2 h of incubation. Lanes 6–8: modified 23-mer without liposomes, with DDAB/DOPE, and with DOTAP after 5 h of incubation. Lanes 9–11: modified 23-mer without liposomes, with DDAB/DOPE, and with DOTAP after 8 h of incubation.


1 2 3 4 5 6 7 8 9 10 11
A
1 2 3 4 5 6 7 8 9 10 11
B
1 2 3 4 5 6 7 8 9 10 11
C

23-mer ODNs into the CaSki cells (Fig. 3a). The increase of cell-associated radioactivity with the liposomes was most prominent after 2 h of incubation. At 2 h the radioactivity of the CaSki cells increased 4.5-times by DDAB/DOPE and 2.0–2.5-times by DOTAP liposomes compared to ODNs without liposomes. The relative efficiency of the cationic liposomes compared to ODNs without liposomes decreased during the incubation time. After 8 h the cell associated radioactivity was only 1.5-fold with liposomes compared to that without liposomes. Results of the liposomal 12-mers were parallel with the liposomal 23-mers (data not shown).

Both cationic liposomes improved the delivery of 23-mer ODNs into the nucleus (Fig. 3b). After 2 h, the nucleus associated radioactivity increased almost 20-times by DDAB/DOPE, and almost 10 times by DOTAP when compared to ODNs in solution. After 8 h, the nucleus-associated radioactivity was approx. 2–3-fold higher with the liposomes, as compared to the non-liposomal ODNs (Fig. 3b). Results of the liposomal 12-mers were similar (data not shown).

With both 12- and 23-mers, 30–40% of the cell-associated radioactivity was found in the plasma membrane, and 50–60% in the cytoplasm, when the liposomes were not used. With DDAB/DOPE and DOTAP, 25–35% and 40–55% of the cellular radioactivity was in the plasma membrane and cytoplasm, respectively. The nucleus contained approx. 25% of the cell- associated radioactivity when delivered by DDAB/DOPE (range from 21.8 to 32.9%), and approx. 20% when delivered by DOTAP (range from 14.1 to 30.9%). Without liposomes 4–15% of the cell-associated radioactivity was present in the nucleus. After 2 h, a 3.5–5.0-times higher fraction of the cellular radioactivity was delivered to the nucleus by the liposomes as compared to that without liposomes. After 8 h, a 1.5–2.0-times higher fraction of the cell-associated radioactivity was present in the nucleus by using the liposomes.

The stability of the ODNs in the culture medium and in the cells increased with the cationic liposomes. In the culture medium (Fig. 4a), only a small fraction of the 23-mers without the liposomes was intact after 2 h of incubation (lane 3), and no intact 23-mers were detected after 5 and 8 h of incubation (lanes 6 and 9). Part of the ODNs delivered with DOTAP were intact after 2 and 5 h of incubation, but none after 8 h (lanes 5, 8, 11). The stability of the ODNs was highest with the DDAB/DOPE. At all times, most of the ODNs were 23-mers (Fig. 4a, lanes 4, 7, 10). Similarly, the stability of the ODNs in the cytoplasm and the nucleus increased with the cationic liposomes, especially with the DDAB/DOPE (Fig. 4b and c). All ODNs without the liposomes were degraded already at 2 h in the cytoplasm and nucleus (Fig. 4b and c, lane 3), while most of the ODNs given with the DDAB/DOPE were intact in the cytoplasm even after 8 h of incubation, and a major fraction of 23-mer was also found in the nucleus (Fig. 4b and c, lane 10). The signals in all fractions (medium, cytoplasm, and nucleus) were more intense from the radioactive ODNs with the DDAB/DOPE than with DOTAP. The results of repeated PAGEs were similar. Also the results with the 12-mers were similar to those obtained with the 23-mers (data not shown).

4. Discussion

The nuclease-resistant phosphorothioate ODNs are considered as promising analogs to the phosphodiester ODNs. However, the thioated ODNs have caused several non-sequence-specific effects in vitro [6–10], and possibly similar problems occur also in vivo. Also the number of stereoisomers of ODNs increase with the number of thioate groups added [34]. For these reasons, only minimal modification of the ODN is advisable. Previously, it was shown that one thioate group at 3′-end protected ODNs against nucleases in cell culture medium [12]. However, according to our results, this modification did not protect ODNs against degradation in CaSki cell cultures (Fig. 2). Thus, this modification is not adequate, and other methods are needed to protect the ODNs against degradation.

The length of the ODN chain effected the cellular uptake so that the delivery of the 12-mers into the CaSki cells was more effective than that of the 23-mers. The thioate group at the 3′-end did not have any significant effect on the cellular uptake.

The delivery of the ODNs was faster and more efficient with the liposomes. However, in our experiments, the DDAB/DOPE and DOTAP did not improve the delivery of ODNs as much as expected from the previous studies, where the cellular uptake increased to 10- to 15-fold [23,26]. One reason could be that different cell type was used. Fast degradation of non-liposomal ODNs in CaSki cell cultures releases radioactive ATP from the ODNs. The cellular uptake assays measured also the delivery of radioactive ATPs, which enter the cells easier than the ODNs. In the case of DDAB/DOPE complexed ODNs, degradation in the culture medium was much smaller, and thus, Figs. 3 and 4 underestimate the difference between the liposomal and non-liposomal delivery. Based on the total radioactivity in the cells (Fig. 3a), and on the electrophoresis results (Fig. 4) DDAB/DOPE delivered more intact oligonucleotides into the cells than oligonucleotide solution did.

Both cationic liposomes potentiated the delivery of the ODNs also into the nuclear fraction. Previous studies have also shown that ODNs delivered by DOTMA were located in nucleus [23,28]. Cumin et al. reported that up to 54% of the radioactivity was found in the nuclei in Chinese hamster ovary cells when DOTMA was used [24]. The problem with a subcellular fractionation is that endosomes and lysosomes might partly contaminate the nuclear fraction, and it is difficult to isolate pure subcellular compartments

from each other [4,35]. Thus the nuclear fraction may be an overestimate, because some non-liposomal [35] and liposomal [36] ODNs are endocytosed and localize in endosomes. Despite this fact, the cationic liposomes increased substantially the stability and delivery of ODNs into this subcellular fraction including the nucleus. Further studies are needed to fully elucidate the intracellular distribution of ODNs, e.g., at electron microscopic level, which is in progress in our laboratory.

The most important finding of our study is that DDAB/DOPE significantly increased the stability of the ODNs in the culture medium, cytoplasm and nucleus as well as the cellular delivery of intact ODNs. Also DOTAP improved the stability and delivery, but less than DDAB/DOPE. Capaccioli et al. compared the cellular uptake and stability of ODNs delivered by DOTAP, DOTMA or dioctadecylamidoglycyl spermine (DOGS) [37]. In this study DOTAP was the most efficient vehicle. On the basis of that and our results, DDAB/DOPE might be more efficient method for intracellular delivery of ODNs. The disadvantage of DDAB/DOPE is that it is more toxic to the cells than DOTAP at higher concentrations (40 μM) [38]. However, according our recent results no difference are seen in the toxicity between DDAB/DOPE and DOTAP at the concentration of 10 μM [38], which is the concentration used in the present study. If the toxicity of DDAB/DOPE does not interfere with the applications, DDAB/DOPE is a better vehicle than DOTAP.

The improved stability in the culture medium indicates that the cationic liposomes per se, protect the ODNs against the enzymatic degradation, and consequently, can deliver intact ODNs into the cells, in contrast to non-liposomal ODNs. After 2 h, less than 10% of the non-liposomal 23-mers were intact in the culture medium (Fig. 4a, lane 3). Consequently less than 10% of the cell-associated radioactivity was derived from the intact 23-mers. The total amount of intact ODNs inside the cells was thus less than 0.05%. Instead, the majority (nearly 100%) of the DDAB/DOPE- delivered 23-mers were intact in all fractions (i.e., the medium, cytoplasm and nucleus) (Fig. 4). The cell-associated radioactivity of the 23-mers was approx. 2.5% with the DDAB/DOPE, indicating that the DDAB/DOPE increased the delivery of intact ODNs over 50-fold into the cells compared to that without liposomes. It is much more than simply indicated in Fig. 3a and b, which show also the radioactivity of degraded ODNs. In fact, no intact ODNs were detected inside the cells after incubation without liposomes. According to the membrane fusion theory, cationic liposomes mostly fuse with the plasma membrane and ODNs are released into the cytoplasm. Intracellular endo- and exonucleases may degrade ODNs in the cells [28]. By increasing the intracellular concentration of intact ODNs, liposomal delivery may prolong the enzymatic half-life of ODNs, because as saturation of enzymes is approached the half-life is increased according to Michaelis-Menten kinetics. Thus liposomes protect ODNs directly in the medium, and possibly indirectly in the cells.

In previous experiments, the cationic liposomes caused 100- or 1000-fold increase in the biological efficacy of antisense ODNs, although the cellular uptake was increased 'only' to 10- to 15-fold [23,26]. On the basis of our study, the increased stability together with the changed intracellular delivery of the ODNs might explain their higher biological activity. The probable potentiated delivery of ODNs into the nucleus is an important advantage, especially when the triple helix techniques are used.

The delivery of ODNs varied in different experiments (Figs. 1a and 3a). This is probably due to the interexperiment variation of cell density, previously shown to influence the delivery of ODNs [39]. Despite this, the delivery of the liposomal ODNs into the cells and nucleus increased in parallel in all experiments compared to that without liposomes. Also the percentage of radioactivity in different cellular fractions was the same in all experiments. Thus, the results within the same experiment can be compared to each other. Comparing the data in Fig. 2 with those in Fig. 4a, shows the effect of oligonucleotide concentration on their stability. All 23-mers without the liposomes were degraded at the concentration of 0.1 μM already in 5 h (Fig. 4a, lane 6), while after 8 h of incubation, part of the 23-mers were intact at the concentration of 5 μM (Fig. 2, lanes 12 and 16). This may be due to the longer half-life of nuclease induced degradation at 5 μM compared to 0.1 μM.

In conclusion, the delivery of the intact ODNs was slow and inefficient without the cationic liposomes. DDAB/DOPE and DOTAP improved the cellular delivery of intact antisense ODNs by protecting them against nucleases in medium, by increasing the total uptake of ODNs in CaSki cells, and by increasing the nuclear localization of ODNs in cells. Only with liposomal delivery intact ODNs were seen in the cytoplasm and nucleus. Of these two cationic liposomes, DDAB/DOPE enhanced both the delivery and stability of the ODNs significantly more than did DOTAP.

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References

[1] Cohen, J.S. (1992) Trends Biotechnol. 10, 87–91.
[2] Crooke, S.T. (1992) Biotechnology 10, 882–886.

[3] Stein, C.A. and Cheng, Y.-C. (1993) Science 261, 1004–1011.
[4] Milligan, J.F., Matteucci, M.D. and Martin, J.C. (1993) J. Med. Chem. 36, 1923–1937.
[5] Stein, C.A., Tonkinson, J.L. and Yakubov, L. (1991) Pharm. Ther. 52, 365–384.
[6] Stein, C.A., Subasinghe, C., Shinozuka, K. and Cohen, J.S. (1988) Nucleic Acids Res. 16, 3209–3221.
[7] Ghosh, M., Ghosh, K. and Cohen, J.S. (1992) Antisense Res. Dev. 2, 111–118.
[8] Cazenave, C., Stein, C.A., Loreau, N., Thuong, T., Neckers, L.M., Subasinghe, C., Helene, C.S., Cohen, J.S. and Tolume, J.-J. (1989) Nucleic Acids Res. 17, 4255–4273.
[9] Yaswen, P., Stampfer, M.R., Ghosh, K. and Cohen, J.S. (1993) Antisense Res. Dev. 3, 67–77.
[10] Watson, P.H., Pon, R.T. and Shiu, R.P. (1992) Exp. Cell Res. 202, 391–397.
[11] Ghosh, M.K., Ghosh, K. and Cohen, J.S. (1993) Anticancer Drug Des. 8, 15–32.
[12] Hug, H. and Wiesner, P. (1992) Methods Mol. Cell. Biol. 3, 156–158.
[13] Shaw, J.-P., Kent, K., Bird, J., Fishback, J. and Froehler, B. (1991) Nucleic Acids Res. 19, 747–750.
[14] Krieg, A.M., Tonkinson, J., Matson, S., Zhao, Q., Saxon, M., Zhang, L.-M., Bhanja, U., Yakubov, L. and Stein, C.A. (1993) Proc. Natl. Acad. Sci. USA 90, 1048–1052.
[15] Clarenc, J.P., Degols, G., Leonetti, J.P., Milhaud, P. and Lebleu, B. (1993) Anticancer Drug Des. 8, 81–94.
[16] Geselowitz, D. and Neckers, L. (1992) Antisense Res. Dev. 2, 17–25.
[17] Yakubov, L.A., Deeva, E.A., Zarytova, V.F., Ivanova, E.M., Ryte, A.S., Yurchenko, L.V. and Vlassov, V.V. (1989) Proc. Natl. Acad. Sci. USA 86, 6454–6458.
[18] Akhtar, S., Basu, S., Wickstrom, E. and Juliano, R.L. (1991) Nucleic Acids Res. 19, 5551–5559.
[19] Loke, S.L., Stein, C.A., Zhang, X.H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J.S. and Neckers, M. (1989) Proc. Natl. Acad. Sci. USA 86, 3474–3478.
[20] Jaroszewski, J. W and Cohen, J.S. (1991) Adv. Drug Deliv. Rev. 6, 235–250.
[21] Thierry, A.R. and Dritschilo, A. (1992) Nucleic Acids Res. 20, 5691–5698.
[22] Thierry, A.R., Rahman, A. and Dritschilo, A. (1992) in Gene Regulation: Biology of Antisense RNA and DNA, pp. 147–161, Raven Press, New York.
[23] Bennet, C.F., Chiang, M.-Y., Chan, H., Shoemaker, J.E. and Mirabelli, C.K. (1992) Mol. Pharmacol. 41, 1023–1033.
[24] Cumin, F., Asselbergs, F., Lartigot, M. and Felder, E. (1993) Eur. J. Biochem. 212, 347–354.
[25] Pelarky, L., Saijo, Y., Busch, R.K, Bennett, C.F., Mirabelli, C.K., Crooke, S.T. and Busch, H. (1993) Anti-Cancer Drug Design 8, 3–14.
[26] Chiang, M-Y., Chan, H., Zounes, M.A., Freier, S.M., Lima, W.F. and Bennett, C.F. (1991) J. Biol. Chem. 226, 18162–18171.
[27] Colige, A., Sokolov, B.P., Nugent, P., Basegra, R. and Prockop, D.J. (1993) Biochemistry 32, 7–11.
[28] Wagner, R.W., Matteucci, M.D., Lewis, J.G., Gutierrez, A.J., Moulds, C. and Froehler, B.C. (1993) Science 260, 1510–1513.
[29] Rose J.K., Buonocore, L. and Whitt, M.A. (1991) Biotechniques 10, 520–525.
[30] Syrjänen, K.J. (1987) In Papillomaviruses and human diseases (Syrjänen, K.J., Gissmann, L. and Koss, L.G., eds.), 1st Edn., pp. 468–503, Springer-Verlag, Heidelberg.
[31] Haigler, H.T., Maxfield, F.R., Willingham, M.C. and Pastan, I. (1980) J. Biol. Chem. 255, 1239–1241.
[32] Gao, W.-Y., Jaroszewski, J.W., Cohen, J.S. and Cheng, Y.-C. (1990) J. Biol. Chem. 265, 20172–20178.
[33] Roop, D.R., Nordström, J.L., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1978) Cell 15, 671–685.
[34] Ghosh, M.K. and Cohen, J.S. (1992) Progr. Nucleic Acid Res. Mol. Biol. 42, 79–126.
[35] Fisher, T.L., Terhorst, T., Xiaodong, C. and Wagner, R.W. (1993) Nucleic Acids Res. 21, 3857–3865.
[36] Legendre, J.-Y. and Szoka, F.C., Jr. (1992) Pharm. Res. 9, 1235–1242.
[37] Capaccioli, S., Di Pasquale, G., Mini, E., Mazzei, T. and Quattrone, A. (1993) Biochem. Biophys. Res. Commun. 197, 818–825.
[38] Lappalainen K., Jääskeläinen, I., Syrjänen, K., Urtti, A. and Syrjänen, S. (1994) Pharm. Res. 11, 1127–1131.
[39] Iwanaga, T. and Ferriola, P.C. (1993) Biochem. Biophys. Res. Commun. 191, 1152–1157.