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Cationic liposomes mediated delivery of antisense oligonucleotides targeted to HPV 16 E7 mRNA in CaSki cells

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Summary

The "high risk" types 16 and 18 of human papillomavirus (HPV) are involved in the etiology of genital squamous cell carcinoma. The early genes 6 and 7 (E6–E7) of these viruses code for the major transforming proteins, capable of inducing cell transformation alone or acting synergistically with other oncogenes. Antisense oligonucleotides, recently applied to inhibit the functions of a number of cellular and viral proteins, might provide the basis for a new therapeutic strategy against HPV-induced malignancies. We studied the proliferation of CaSki cells by the MTT assay after their exposure to HPV 16 E7 mRNA antisense oligonucleotides with and without cationic liposomes (containing dimethyldioctadecylammonium bromide DDAB, and dioleylphosphatidylethanolamine, DOPE). Unmodified oligonucleotides (either 12- or 23-mers) did not have any effect on either CaSki cell proliferation or morphology when compared with the untreated cells. The cellular uptake of oligonucleotides was significantly enhanced by the cationic liposomes as assessed by confocal laser scanning microscopy (CLSM). The cationic liposomes were toxic to the cells as demonstrated by the reduced cell number and altered cell morphology. Only a slight reduction of the cell proliferation was seen when antisense 12-mer was protected from its 3′- and 5′-ends with thiolate and FITC, respectively. Both the 12- and the 23-mers with the cationic liposomes inhibited

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cell proliferation, the inhibitory effect being longer with the 23-mer. Overall, the MTT assay was less sensitive than light microscopy to reveal the toxic effects on CaSki cells. The results suggest that antisense oligonucleotides targeted to HPV 16 E7 mRNA can be introduced into CaSki cells with cationic liposomes.

HPV; Antisense oligonucleotides; Cationic liposomes; CaSki cells

Introduction

The etiological role of human papillomaviruses (HPV) in the development of cervical cancer has been suspected since 1974 (zur Hausen et al., 1974). The HPV family consists of increasing number of different types, 68 having been recognized by now (Falcinelli et al., 1992). Convincing epidemiological, clinical and experimental data support the concept that high-risk HPV types are causally involved in the pathogenesis of anogenital cancer, that of the uterine cervix, in particular. In large series analysed, viral DNA was found in about 90% of such tumours, being most frequently of HPV type 16 or 18 (zur Hausen, 1991).

HPVs are small (52–55 nm diameter), double-stranded DNA viruses that replicate in the nucleus of squamous epithelial cells (Syrjänen, 1987; Syrjänen, 1992). All HPVs have a closed circular, double-stranded DNA genome about 8.000 base pairs long (Syrjänen, 1987; Chang, 1990; Syrjänen, 1992). Protein-coding sequences (ORFs) are classified as early (E) or late (L) genes. The biological functions of most ORFs have been identified, E6–E7 ORFs coding for the major transforming proteins (Syrjänen, 1987; Chang, 1990; Syrjänen 1992).

E6–E7 genes of the high-risk HPV types immortalize human foreskin or cervical keratinocytes (Münger et al., 1989). In organotypic cultures, these immortalized cells resemble intraepithelial neoplasias in their growth characteristics (Woodworth et al., 1990). Although HPV E6–E7-immortalized human cells are initially nontumorigenic in nude mice, long time in vitro cultivation may lead to malignant clones (Pecoraro et al., 1991). The development of specific means to inhibit these transforming functions of the HPV E6 and E7 genes might provide the basis for a new therapeutic strategy against, e.g., cervical cancer (Falcinelli et al., 1992).

Oligonucleotides designed to hybridize to spesific mRNA sequences (antisense oligonucleotides) have been recently applied to inhibit the functions of a number of cellular and viral proteins. These oligonucleotides are suggested to be applicable in the treatment of various viral diseases and virus-associated cancer as well (Hélène and Toulmé, 1990; Cohen, 1992; Crooke, 1992; Ghosh and Cohen, 1992). One of the major problems in their practical applications is the fact that unmodified oligonucleotides have poor biological stability due to

degradation by nucleases. To circumvent these obstacles, a variety of modified and more stable oligonucleotide analogues have been developed including phosphorothioate oligonucleotides (*S*-oligos). However, even these modified oligonucleotides may be degraded relatively quickly (Crooke, 1992; Stein et al., 1992). Another reason for the high concentrations required to be effective is the poor cellular uptake of oligonucleotides due to their size and negative charge (Crooke, 1992; Stein et al., 1992). In addition, the cell type, components of the media, length of oligonucleotide as well as the presence of linked groups affect the cellular uptake of oligonucleotides (Jaroszewski and Cohen, 1991). Intracellular delivery and efficacy of oligonucleotides and DNA have been increased significantly with cationic liposomes (Felgner et al., 1987; Bennett et al., 1992; Legendre and Szoka, 1992).

There are recent studies where antisense oligonucleotides are targeted to the mRNA of E2, E6 and E7 genes of papillomaviruses (Storey et al., 1991; Cowsert et al., 1993; Steele et al., 1993). In these studies antisense oligonucleotides showed specific effects on the cells. However, none of these studies have used carrier to improve the cellular uptake of the oligonucleotides. In the present study the specific aim was to investigate the cellular uptake of oligonucleotides with cationic liposomes. We have investigated the proliferation of CaSki cells after the treatment with oligonucleotides targeted at E7 mRNA of HPV 16 using a colorimetric MTT assay. CaSki cells, originally derived from human cervical cancer, contain approximately 600 copies of HPV 16, and the E7 gene is continously expressed in these cells. We compared also the effects of unmodified and modified 12-mer and 23-mer. The delivery to CaSki cells was studied using fluorescein-isothiocyanate (FITC)-labelled oligonucleotides by confocal laser scanning microscope (CLSM).

Materials and Methods

Cell culture

CaSki cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland, UK) at 37°C in 5% CO_2. MTT assay was performed in 96-well plates (Nunc, Roskilde, Denmark) with 10^4 cells/well. For morphological and CLSM studies 5.2×10^4 cells/well were seeded onto round glass microscope slides in 24-well plates (Nunc, Roskilde, Denmark). Oligonucleotides, liposomal oligonucleotides and liposomes were added 12 h later and incubated for 1, 2, 3 or 4 days. All assays were performed in triplicate.

Oligonucleotides

In the present study, seven different oligonucleotides were used as summarized in Table 1. The target site of the oligos was the initiation region of the HPV 16 E7 ORF. As a control, the part of the sequence from the gene encoding the mouse growth hormone (12-mer random oligo) was used. This

TABLE 1
Molecular characteristics of oligonucleotides used

Description of oligonucleotide	Symbol	Length (nt)	Target	Sequence (5′–3′)
Unmodified phosphodiester	E7-12	12	E7	ATCTCCATGCAT
Phosphorothioate	S-E7-12	12	E7	ATCTCCATGCAT#
Phosphorothioate	S-E7-F-12	12	E7	*ATCTCCATGCAT#
Unmodified phosphodiester	E7-23	23	E7	AATGTAGGTGTATCTCCATGCAT
Phosphorothioate	S-E7-F-23	23	E7	*AATGTAGGTGTATCTCCATGCAT#
Unmodified phosphodiester	RAND-12	12	no specific target	ACCAATCGTTCC
Phosphorothioate	S-RAND-F-12	12	no specific target	*ACCAATCGTTCC#

* and # indicates the site of FITC-label and thiolate-group, respectively.

sequence has no homology with human DNA. Antisense or random oligodeoxynucleotides and their structural analogs were synthesized by an automated DNA synthesizer (Gene Assembler Plus, Pharmacia, Uppsala, Sweden). A thiolate group (Antisense Reagent, Pharmacia, Uppsala, Sweden) and FITC-label (FluorePrime, Pharmacia, Uppsala, Sweden) were added to the 3′-end and the 5′-end, respectively. All oligonucleotides given in Table 1 were used in studies to assess the cell morphology and proliferation. FITC-labelled oligonucleotides were applied to the CLSM experiments. The final concentrations of oligonucleotides and liposomal oligonucleotides in the medium were 10 μM and 5 μM, respectively.

Liposomes

A cationic liposomal preparation containing dimethyldioctadecylammonium bromide (DDAB) and dioleylphosphatidylethanolamine (DOPE) (2:5, w/w) was used, as described previously (Rose et al., 1991). DDAB (1.32 mg) and DOPE (3.31 mg) in chloroform were evaporated to dryness in a rotating evaporator. 3 ml of sterile water were added, and after 1 h, the mixture was sonicated for 10 min (Branson bath sonicator, Model 2200, Danbury, CT, USA). The size was determined by quasi-elastic light-scattering (Nicomp Submicron Particle Sizer, Model 370, Santa Barbara, CA). The mean size of vesicles was 52.6 nm with a standard deviation of 40.9 nm. Before adding to the cells, DDAB:DOPE was incubated with oligonucleotides for 10 min at room

temperature. In all experiments the final concentration of liposomes was 10 μM in culture medium.

Morphology

CaSki cells were washed twice with phosphate-buffered saline (PBS), fixed in 4% formaldehyde and stained with toluidine blue for routine light microscopy.

CLSM

For CLSM, the cells were washed in PBS, and fixed in 4% formaldehyde. The cellular uptake of FITC-labelled *S*-oligos with or without liposomes was analyzed using the Leica CLSM with argon laser (Leica Lasertechnik, Heidelberg, Germany). The excitation wavelength was 488 nm, and a 515 nm long pass filter was used for emission. In comparing the different treatments, the adjustments of CLSM (offset, voltage and pinhole) were kept constant.

MTT assay

MTT assay is a rapid colorimetric assay to measure cellular growth and survival (Mosmann, 1983; Denizot and Lang, 1986). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Amresco, Ohio, USA) assay measures the activity of the mitochondrial dehydrogenases of viable and proliferating cells. The dehydrogenases of cells reduce MTT to a blue formazan product, that can be measured spectrophotometrically (Mosmann, 1983; Denizot and Lang, 1986; Keisari, 1992). The MTT assay was performed after 1, 2, 3 and 4 days incubation with oligonucleotides. MTT was dissolved in PBS at 5 mg/ml and immediately before use diluted to 1 mg/ml of the culture medium. The solution was filtered through a 0.22 μm filter to remove formazan crystals. Culture medium was removed from the cells, and diluted MTT solution (100ul) was added. The cells were incubated for 3 h at 37°C. Then MTT solution was removed and acid-isopropanol (100 μl of 0.04 M HCl in isopropanol) was added. Cells were shaken for 20 min at room temperature to ensure that all crystals were dissolved. Plates were read on a multiwell scanning spectrophotometer (Labsystems Multiskan^{R} Plus ELISA reader, Helsinki, Finland) using the wavelength of 570 nm.

Results

Morphology

The morphologic alterations of the CaSki cells after a 4-day incubation with oligonucleotides are summarized in Fig. 1. DDAB:DOPE at 10 μM was highly toxic to the cells (1a). The cells were more intensely stained, the cell number and the cell size were remarkably reduced. Similar changes, albeit less marked, were found when 5 μM oligonucleotides with 10 μM DDAB:DOPE were added (Fig. 1b). Changes of the cell morphology were clearly seen even after a 1-day incubation with liposomes, and these changes were accentuated during the

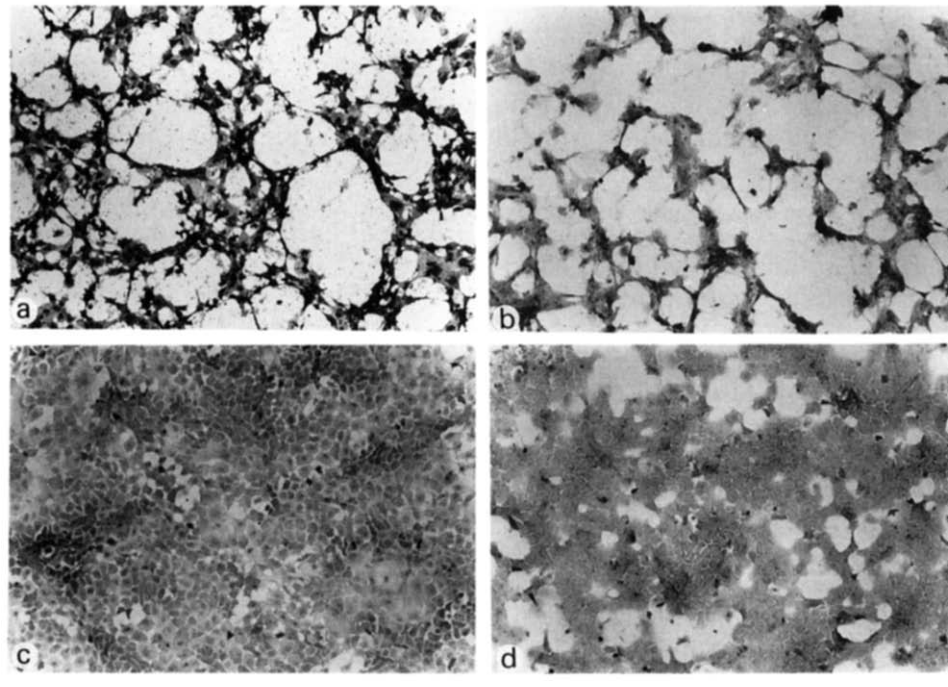

Fig. 1. Morphology of the cells after 4 days of treatment with oligonucleotides and liposomes (original magnification × 100): (a) DDAB:DOPE, (b) S-E7-F-12mer with DDAB:DOPE, (c) S-E7-F-12mer, (d) the control: CaSki cells without any treatment.

experiment. As shown in Fig. 1c, oligonucleotides without cationic liposomes had no effect on CaSki cell morphology. Caski cells without any treatment are shown in Fig. 1d.

CLSM

In general, FITC-labelled *S*-oligos were efficiently introduced into the cells with DDAB:DOPE (Fig. 2b,2c) but marked variation in the cellular uptake was observed within individual samples. The cellular penetration of oligonucleotides without liposomes (Fig. 2a) was significantly lower than DDAB:DOPE complexed oligonucleotides. In the absence of DDAB:DOPE, the oligos appeared to be associated with the cell membrane, whereas the oligos with DDAB:DOPE were also found in the cytoplasm (Fig. 2b,c). Autofluorescence of the CaSki cells was lower than fluorescence of FITC (Fig. 2d).

MTT assay

In preliminary experiments, we investigated the relationship between the MTT absorbance values, cell number and the optimal seeding density of CaSki cells. In these experiments the linear relationship between formazan produced

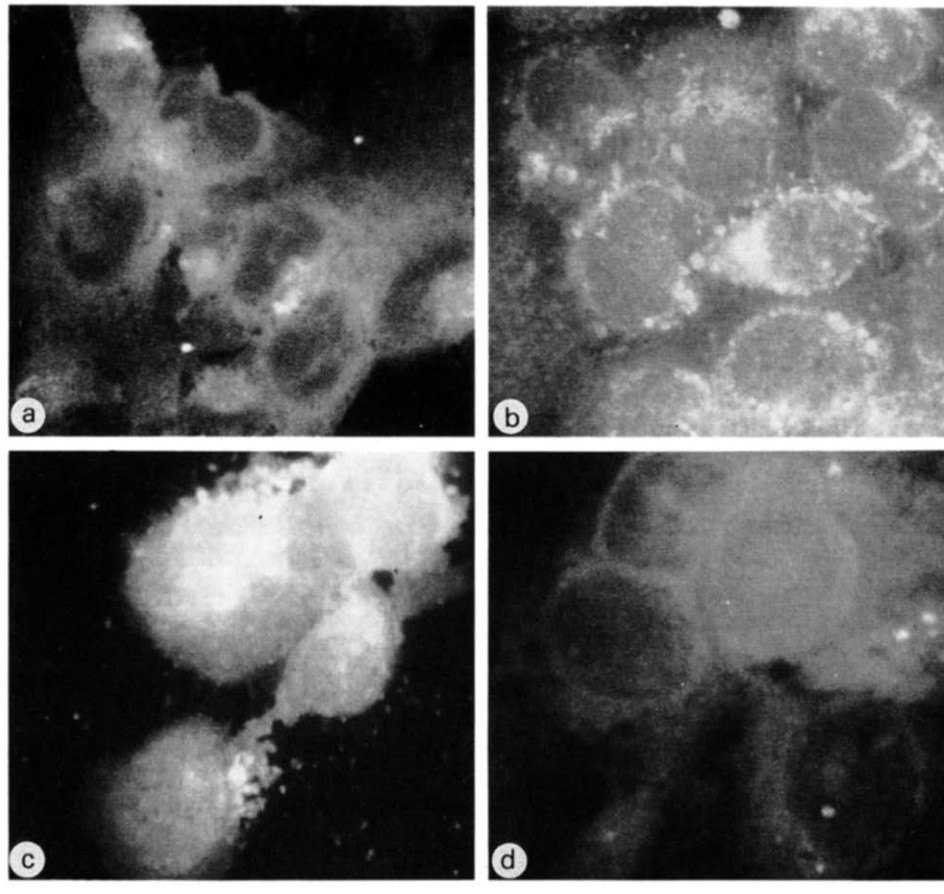

Fig. 2. CLSM images. (a) CaSki cells treated with antisense S-E7-F-12-mer for 1 day. Voltage: 713, offset: −27, step 0.8 μm, objective 60.0'/1.3–0.6 oil, zoom 1.0. (b) CaSki cells treated with liposomal antisense S-E7-F-12-mer for 1 day. The adjustments were same as in Fig. 2a. (c) CaSki cells treated with liposomal S-rand-F-12-mer for 1 day. Voltage 630, offset 29, step 1.0 μm, objective 40/0,70 T oil, zoom 1.6. (d) CaSki cells cultivated for 1 day without any treatment. The adjustments were same as in Fig. 2c.

in MTT assay and cell number was observed, when the seeding density was 10^4 CaSki cells per well and the cells were incubated from 1 to 4 days. In preliminary experiments the MTT assay was used to determine the optimum concentrations of DDAB:DOPE and the oligonucleotides. DDAB:DOPE was shown to be too toxic at concentrations of over 10 μM. Oligonucleotides (both anti- or random) had no toxic effects at the concentrations below 50 μM (data not shown).

The results of the MTT assays are summarized in Fig. 3. Fig. 3a shows that neither a random 12-mer nor antisense E7-12-mer had any effect on the proliferation of CaSki cells. CaSki cells without any treatment showed a

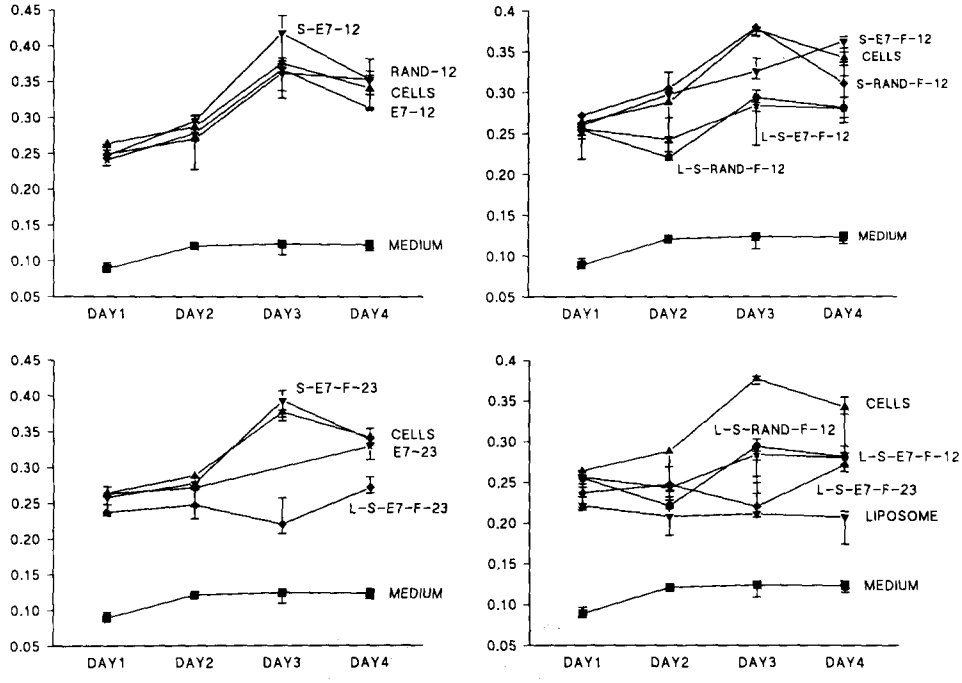

Fig. 3. Results of the MTT assay. A single experiment was carried out in a triplicate. Each point indicates the medium value of three measurements. The error bars represent the range of the results. The vertical (y) axis shows the optical density values of MTT assay. Before incubations cells were first grown for 12 h. The cells were incubated 1, 2, 3 and 4 days with oligonucleotides and liposomes. Cells indicate CaSki cells without any treatment, L indicates liposomal oligonucleotides and the symbols of oligonucleotides are the same as given in Table 1. Medium (no cells, no oligonucleotides) presents the background caused by the cell culture medium.

proliferation during the first 3 days, but an inhibition of the proliferation was seen at day 4 due to the contact inhibition. When the 3'-end of the E7–12 oligonucleotide was protected with a thiolate group, a slight induction of the cell proliferation was seen.

Neither antisense S-E7-F-23-mer nor E7–23-mer had any effect on the cell growth as assessed by MTT assay (Fig. 3b). The missing values of E7–23-mer at day 3 were due to a technical error. In Fig. 3c it is shown that antisense S-E7-F-12-mer caused only a slight reduction at day 3 of the cell proliferation. Unmodified random-12-mer and modified S-random-F-12-mer did not have any effect on the cell growth (Fig. 3a and c).

Fig. 3d shows the obvious toxic effect of cationic liposomes on CaSki cells at a concentration of 10 μM. When oligonucleotides were incubated with liposomes, the toxic effects were slighter than those seen with DDAB:DOPE alone. Although the retardation of cell proliferation was found with all liposomal oligonucleotides, there were differences between 12- and 23-mers: the

12-mer inhibited cell proliferation during the first 2 days, while the 23-mer showed inhibition during 3 days. There was no difference between liposomal S-random-F-12-mer and liposomal S-E7-F-12-mer. Subsequent proliferation occurred for all liposomal oligonucleotides. This proliferation was not observed when the cells were treated with the pure cationic liposomes.

Discussion

So far, no specific chemoterapeutic agents are available to eradicate HPV infections in the squamous cells. The use of interferons (as a monotherapy) in a large number of clinical trials has proved unsuccessful (Syrjänen, 1987), and effective vaccines are not available. Thus, an urgent need exists to develop new therapeutic agents applicable to eliminate the virus in the genital (and other) mucosal sites. Antisense oligonucleotides seem to be a promising new tool in experimental therapy against, e.g., viral infections. They can be used as inhibitors or regulators of specific viral genes involved in cell transformation or in productive infections. Future progress in the antisense therapy depends largely on the development of applicable delivery systems for the oligonucleotides into the cells.

In our study, the use of cationic liposomes (DDAB:DOPE) increased significantly the cellular uptake of oligonucleotides. This is in agreement with the recent data from other groups using cationic liposomes (Akhtar et al., 1991; Bennett et al., 1992; Thierry et al., 1992a). However, cationic liposomes proved to be toxic to the cells, as evidenced by the severe morphological changes in CaSki cells (Fig. 1). In the MTT assay no significant changes could be found after 1-day incubation despite the altered cell morphology. This indicates that the light microscopic morphology is more sensitive than the MTT assay in showing the toxic effects of DDAB:DOPE on CaSki cells. MTT assay measures mitochondrial dehydrogenases of viable cells. Mitochondria are known to be the last organelles to survive after cell injury that might explain the discrepancy between light microscopy and the MTT assay. The present results clearly indicate that the examination of cell morphology is mandatory in evaluating experiments with antisense oligonucleotides.

The initiation region of HPV 16 E7 ORF was selected as the target site of the antisense oligonucleotides. Several previous studies have shown that blocking the initiation region of a desired gene will result in a significant inhibition of the gene expression (Vickers et al., 1991; Nobuhiko and Tokuhisa, 1992). However, contradictory results also exist (Felgner et al., 1987). In accordance with previous studies (Byrn et al., 1991) we found also that 12-mers were more effective than 23-mers. However, the inhibition was quite slight and only seen for the modified antisense S-E7-f-12-mer. Modification of the 12-mer in its both ends might prevent its degradation, which could explain the slight inhibition of proliferation on CaSki cells. No specific antisense effect was seen with the similarly modified 23-mer. This might be due to the size of a 23-mer,

which prevents the effective cellular uptake. One explanation, why antisense S-E7-12-mer did not cause any reduction when compared to the untreated control cells at day 4, might be the contact inhibition of the control cells resulting in decreasing MTT value. The cells treated with the unmodified random or the modified random oligonucleotides grew similarly as the control cells.

The oligonucleotides are not entrapped into the cationic liposomes, but they are complexed by electrostatic interaction between the negative oligonucleotides and the positive charges on the liposomes (Thierry et al., 1992b). Thus oligonucleotides mask the positive charges and simultaneously the toxicity of the cationic liposomes (Fig. 3d). We have used the 12- and 23-mers in equivalent molar ratio. Since the number of negative charges in 23-mer is almost twice that of the 12-mer, one might hypothesize that cationic liposomes with an equivalent molar amount of the 12-mer are more toxic than of the 23-mer. However, the results pointed to the opposite: the antisense S-E7-F-23-mer with liposomes inhibited cell proliferation for a longer time than the antisense S-E7-F-12-mer with liposomes. This finding might indicate that cationic liposomes might improve cellular uptake of longer oligonucleotides.

The slight decrease of the proliferation found by antisense S-E7-F-12-mer and the liposomal antisense S-E7-F-23-mer might be due to the specific inhibition of E7 gene as shown recently by Storey et al. (1991). This hypothesis can be confirmed only by assessing the expression level of E7 gene, which was not done in this study. The role of E7 gene in cell transformation is well established, but its association to cellular proliferation is not well known and needs further studies.

Recently, a study on CaSki cells treated with antisense oligonucleotides showed that phosphorothioate (in 3′ end) 21-mer targeted at the initiation region of HPV 16 ORF E7 at a concentration of 5 μM inhibited [^{3}H]thymidine uptake and decreased CaSki cell proliferation (Storey et al., 1991). In our study phosphorotioate (in 3′ end) 23-mer without cationic liposomes did not inhibit the cell proliferation. These contrasting results might be explained by the different assays used. We decided to select the MTT assay instead of the conventional [^{3}H]thymidine assay, because the nonspecific intracellular degradation of the added oligonucleotides may cause a release of thymidine and consequently a competition of the binding with [^{3}H]thymidine during the proliferation assays (Krieg, 1993).

Oligonucleotides with liposomes decreased CaSki cell proliferation at the onset, but after 2 or 3 days, a distinct stimulation was seen. This could signify a recovery of CaSki cells after the toxic effects of liposomes. Evidence of such a recovery was not seen, however, when the morphology of CaSki cells was monitored by light microscopy.

Mechanisms of the cationic liposome-mediated delivery of oligonucleotides into the cells are not clearly understood. Both active and passive transport have been implicated (Jaroszewski and Cohen, 1991). It is known that positively charged liposomes fuse with the plasma membrane (Felgner et al., 1987). The

oligonucleotides with the cationic liposomes can be also taken up into the cells by endocytosis (Legendre and Szoka, 1992). Recently it was shown that oligonucleotides with phosphatidyl-based non-fusing liposomes were mainly localized in intracytoplasmic vesicles (Akhtar et al., 1991). As observed on our CLSM figures, the oligonucleotides with DDAB:DOPE in cells might support the hypothesis of fusion with the plasma membrane. However, further studies are needed to elucidate the transport mechanisms.

Undoubtedly, the access of the antisense oligonucleotides into their target cells at adequate concentrations is a prerequisite for their use as applicable therapeutic agents. Thus, new means to improve the uptake of oligos into the cells are likely to represent a step forward to their successful clinical applications. As shown by the present results, cationic liposomes facilitating the intracellular delivery of antisense oligos seem a promising approach. Even the toxic effects of liposomes might be a benefit for topically applied antisense oligos in eradicating virus in infected cells.

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