Nuclear Transport of Oligonucleotides in HepG2-Cells Mediated by Protamine Sulfate and Negatively Charged Liposomes

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Purpose. The aim of this study was to characterize the intracellular fate and nuclear uptake kinetics of oligonucleotides (ON) that were complexed with protamine sulfate (PS) and negatively charged liposomes at different ratios of ON to PS.

Methods. Double-fluorescence labelling of ON and liposomal lipid was applied to simultaneously monitor the interaction as well as the individual fate of active agent and carrier upon intracellular delivery using confocal laser scanning microscopy (CLSM). A DNA-analogue of a 68-mer intramolecular double-stranded RNA:DNA-hybridoligonucleotide (chimeraplasts) with unmodified phosphate backbone was employed. This construct was condensed with PS and coated with a liposomal formulation (AVE™-3 = artificial viral envelope).

Results. PS–ON complexes and AVE™-3-coated complexes with a defined composition were very effective in nuclear transport of ON for a ON:PS charge ratio of 1:3. Nucleus:cytosol fluorescence ratios peaked at about 10 hrs and started to decrease again at 21 hrs.

Conclusions. AVE™ associates with PS-condensed ON, and this complex is able to be taken up by cells and to deliver ON to the nucleus. PS–ON complexes are released from the liposomal formulation, mainly as an extranuclear enzymatic degradation of the liposomal phospholipids. The results of the kinetic analysis can be used to optimize transfection protocols with ON in HepG2 cells.

KEY WORDS: oligonucleotide; protamine sulfate; artificial viral envelope; confocal laser scanning microscopy.

INTRODUCTION

Recently, 15- to 28-mer oligonucleotides (ON) have been employed which interact with target DNA or RNA in the nucleus or cytoplasm of the cell (1). More recently, Kmiec and co-workers (2,3) proposed to use double-stranded 68-mer chimeric RNA:DNA-ON to target genomic DNA in the nucleus with the aim of repairing single point mutations as they occur in various genetic diseases such as sickle cell anemia, among others. Whereas for antisense strategy a cytoplasmic localization of ON may be sufficient, repairing of genome point mutation requires nuclear transport of the genetic constructs. The ON employed in this study is a 68-mer molecule with two T-hairpin-loops and complementary base structures, thus forming an intramolecular double-strand as shown in Fig. 1.

Cellular uptake of ON is highly inefficient, as their net negative charge and high molecular weight prohibit efficient transfer across cell membranes without the help of carrier systems (4). When ON were applied to cells as a solution, they were only found in the cytoplasm, whereas after complexation with cationic lipids (DOTMA, DOTMA/DOPE, or DOTAP) (5) or microinjection of fluorescently labeled ON, localization in the nucleus as well as diffuse localization in the cytoplasm was observed (6).

Although cationic liposomal formulations are widely used as gene delivery systems, they lack the transfection efficiency of viral vectors. In addition, they interact nonspecifically and highly promiscuously with (negatively charged) vascular walls, as well as serum components. Alternative carrier systems that are being explored include anionic liposomes, pH-sensitive liposomes, immunoliposomes, or fusogenic liposomes to deliver ON or modified ON into cells (1). Artificial viral envelopes (AVE™) are liposomal formulations with a composition that is derived from the composition of viral envelopes (7). For instance, the HIV-1 viral envelope is composed of approximately equimolar amounts of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin with an equimolar ratio of cholesterol (Cho) to total phospholipid (8). Artificial viral envelopes (AVE) have fusogenic properties, allowing them to transport encapsulated or associated drugs into cells (9).

Anionic liposome delivery systems are less cytotoxic to cells *in vitro* than synthetic cationic gene delivery systems (unpublished results). However, in order to achieve acceptable encapsulation/complexation efficiencies, ON are either required to be complexed with cationic agents such ADDIN as Ca^{2+} (10), or need to be encapsulated into the aqueous interior of fusogenic or pH-sensitive liposomes (11). Complexation with cationic agents, e.g., poly-L-lysine (12,13) causes DNA to be neutralized and to collapse into a compact colloidal structure which can be efficiently coated and taken up into cells. Another promising condensing agent is protamine, which packs DNA in sperm cells. Protamine can be harvested from several fish species and is available in pharmaceutical grade commercially (14). Protamine has been reported to enhance cellular uptake of RNA and DNA (15–17) and, more recently, has been shown to enhance the effectiveness of cationic lipid-mediated transfer of plasmids (18).

In our study, we have condensed an ON with PS and coated this complex with a negatively charged rhodamine-labeled liposomal formulation (AVE™-3). Cellular distribution of the ON and the carrier, with a wide range of PS-DNA charge ratios, was investigated in HepG2 cells following the kinetics of this process using confocal laser microscopy in

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ABBREVIATIONS: AVE, artificial viral envelope; CLSM, confocal laser scanning microscopy; Rh-DOPE, Lissamine™-rhodamine B-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; triethylammonium salt; DOTMA, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride; DOPE, dioleoylphosphatidylethanolamine; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chlorid; EPC, egg-phosphatidylcholine; FCS, fetal calf serum; ON = oligonucleotide; PBS, phosphate buffered saline; PS, protamine sulfate.

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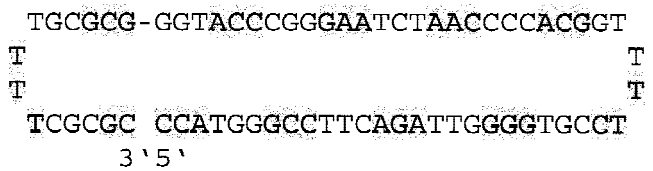

Fig. 1. Structure of the employed DNA–DNA oligonucleotide. Two 4T-hairpin loops flank each side of the molecule. Intramolecular base-pairing is achieved by complementary base positioning.

order to find the optimal formulation for nuclear delivery of oligonucleotides.

MATERIALS AND METHODS

DNA-analogues of chimeric ON as the one used here (Fig. 1) will be referred to as DNA:DNA-ON. A 5′-carboxy-fluorescein-(5′-FAM, obtained from PE Applied Biosystems, Foster City, California, USA) labeled 68-mer DNA:DNA-ON was synthesised and purified with a standard procedure (MWG-Biotech AG, Ebersberg, Germany). ON content was determined spectrophotometrically using a BioPhotometer (Eppendorf-Hamburg, Germany) with a factor of 30 OD/μg ON. The 260/280 ratio was in the range of 1.8 to 2.0.

DOPS was obtained from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Cholesterol was purchased from Calbiochem (La Jolla, California, USA). DLPE was obtained from Sygena (Liestal, Switzerland). 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Rh-DOPE) was from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). The formulation of AVE™-3 and protamine sulfate (Eli Lilly, Indianapolis, Indiana, USA) were a gift of F. Sorgi (Optime Therapeutics, Petaluma, California, USA). EggPC was obtained from Lipoid (Ludwigshafen, Germany).

Preparation of Liposomes

AVE™-3 is composed of equimolar amounts of DLPE, DOPS, and Cholesterol. The lipids were dissolved in chloroform. The solvent was removed by rotary evaporation and the lipid film dried in vacuum for up to 1 hour until all traces of chloroform were removed. After rehydration in degassed 10 mM Tris pH 7.5, the multilamellar vesicles were agitated for 45 min. The multilamellar vesicles were reduced in size to 200–300 nm by gentle ultrasonic treatment using a Soniprep MSE 150 ultrasonic disintegrator with a titanium tip (N. Zivy & CIE SA, Oberwil-Basel, Switzerland) for preparation of AVE™-3. Tip-treated liposomes were centrifuged (5 min, 14000 rpm) in an Eppendorf table centrifuge (Eppendorf, Hamburg, Germany) to remove titanium shedding. Neutral liposomes composed of EggPC and Cholesterol (2/1 molar ratio) were prepared in a similar way. Liposomes were extruded through 50 nm diameter polycarbonate membranes using the LiposoFast™ extrusion device (Avestin, Ottawa, Canada). The size of the liposomes was determined within two hours of extrusion with a Zetasizer 4 (Malvern Instruments, Herrenberg, Germany). In general, liposomes were in the range of 100 nm in diameter (mono-modal analysis).

ON and liposomes were appropriately diluted in 10 mM Tris pH 7.5 in such a way that 20 μl PS-solution was added to 80 μl of DNA-solution, containing 5 μg of DNA:DNA-ON. The complexes were incubated for 30–45 min. Appropriately diluted liposomes were added to the DNA-protamine sulfate (PS)-complexes to give a final volume of 200 μl. After incubation for 15 min the final transfection system, composed of DNA:DNA-ON, PS and liposomes having a size of approx. 150 nm as estimated by electron microscopy (data not shown) was diluted to 1.5 ml with TN-buffer (10 mM Tris pH 7.5/140 mM NaCl) and transferred to HepG2 cells.

PS-ON-complexes and control experiments with naked ON were prepared in the same way.

Cell Culture and Transfection

HepG2 cells were cultured in 6-well-plates. 10^5 cells per well were seeded 5–7 days before transfection and incubated at 37°C and 5% CO_2. Cells were grown in MEM-medium with Earle's salts (Life Technologies GmbH, Karlsruhe, Germany), supplemented with 10% FCS, glutamine, and penicillin–streptomycin solution (Sigma-Aldrich Co. LTD, Irvine, United Kingdom). Cells were washed with PBS immediately prior to the addition of 1.5 ml transfection system. If the incubation time was >5 hours, cells were washed after 3 hours with PBS, and fresh serum-containing medium was added. Cells were incubated at 37°C and 5% CO_2. Postincubation, cells were washed with PBS and transferred to coverslips on which 1 drop of mounting medium was placed. The mounting medium was composed of 22% polyvinylalcohol, 11% Glycerol, 56 mM Tris pH 7.5, and 5% Diazabicyclo[2.2.2]octane, which serves as anti-fading agent (19).

Confocal Laser Scanning Microscopy

A MRC-1000 confocal laser scanning imaging system (Bio-Rad, Hemel Hempstead, United Kingdom) with a Krypton/Argon laser was used. The system was equipped with a Nikon inverted microscope. A 60X oil-immersion objective was used. The system was controlled with Bio-Rad's COMOS-software. Images were converted to TIF-format with Confocal Assistant 4.02 software (Bio-Rad). Integration analysis of images was employed with Scion Image Release Beta 3b (Scion Corporation, Frederick, Maryland, USA).

RESULTS

The uptake of naked ON over the entire observation period of 1 to 21 hours is rather weak as only small amounts of fluorescence label can be found in the cytoplasm of the cells and appears to be attributed to granule structures (Fig. 2). No fluorescence was detectable in the nucleus.

Uptake of the PS–ON complexes is a kinetically controlled process. After 1 hour, only small amounts of fluorescence are observed in the nucleus. Negatively charged PS–ON complexes (at as PS:ON charge ratio of 0.5) show specific nuclear transport of genetic material (Fig. 3a). Over time, fluorescence intensity diminishes (Fig. 3 b, c), indicating that protection from degradation by nucleases may be insufficient, due to the excess of negative charges of the complex. With increasing amounts of PS, nuclear transport of ON is improved. Neutral complexes (Fig. 3 d–f) are able to transport ON to the nucleus with a relative maximum of fluorescence intensity seen after 5 hours of incubation. At a charge ratio of ¾ (+/–) PS:ON, all visible nuclei appear highly fluorescent after 5 hr (Fig. 3 h). Fluorescence decreases after 21 hours,

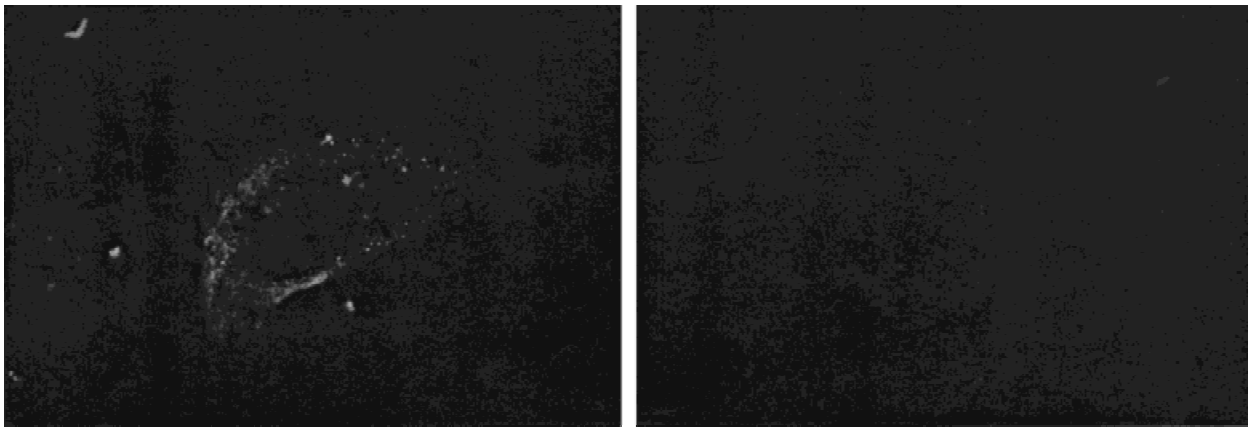

Fig. 2. Cellular uptake of fluorescence-labeled DNA–DNA oligonucleotides without addition of liposomes and protamine sulfate after (a) 1 hour and (b) 21 hours of incubation. Contrast of the image was enhanced using contrast stretch in Confocal Assistant 4.02 software.

independent of the charge ratio of PS to ON (Fig. 3) indicating an as yet undetermined metabolic or elimination processes.

Bright spots which are hardly associated with cellular structures are visible in neutral and cationic preparations (Fig. 3 d–i). These spots are protamine-condensed complexes of ON which interact with the anionic background of collagen treated coverslips. The bright fluorescence is a marker for intact complexes of ON and PS. Cell-associated spots are only found in the border of cells. The complexes are adsorbed to cells by electrostatic interaction or are endocytosed vesicles with still unreleased ON. Besides cell-associated bright spots, there is an even distribution of ON-fluorescence in the cytoplasm, and especially in the nucleus of cells, indicating that the structure of complexes has changed, perhaps due to intracellular endosomal digestion of PS, with release of ON into the cytoplasm or nucleus of the cells. Nuclear fluorescence of ON is always smooth, indicating release of ON from complexes.

Uptake and cellular distribution of PS-condensed ON

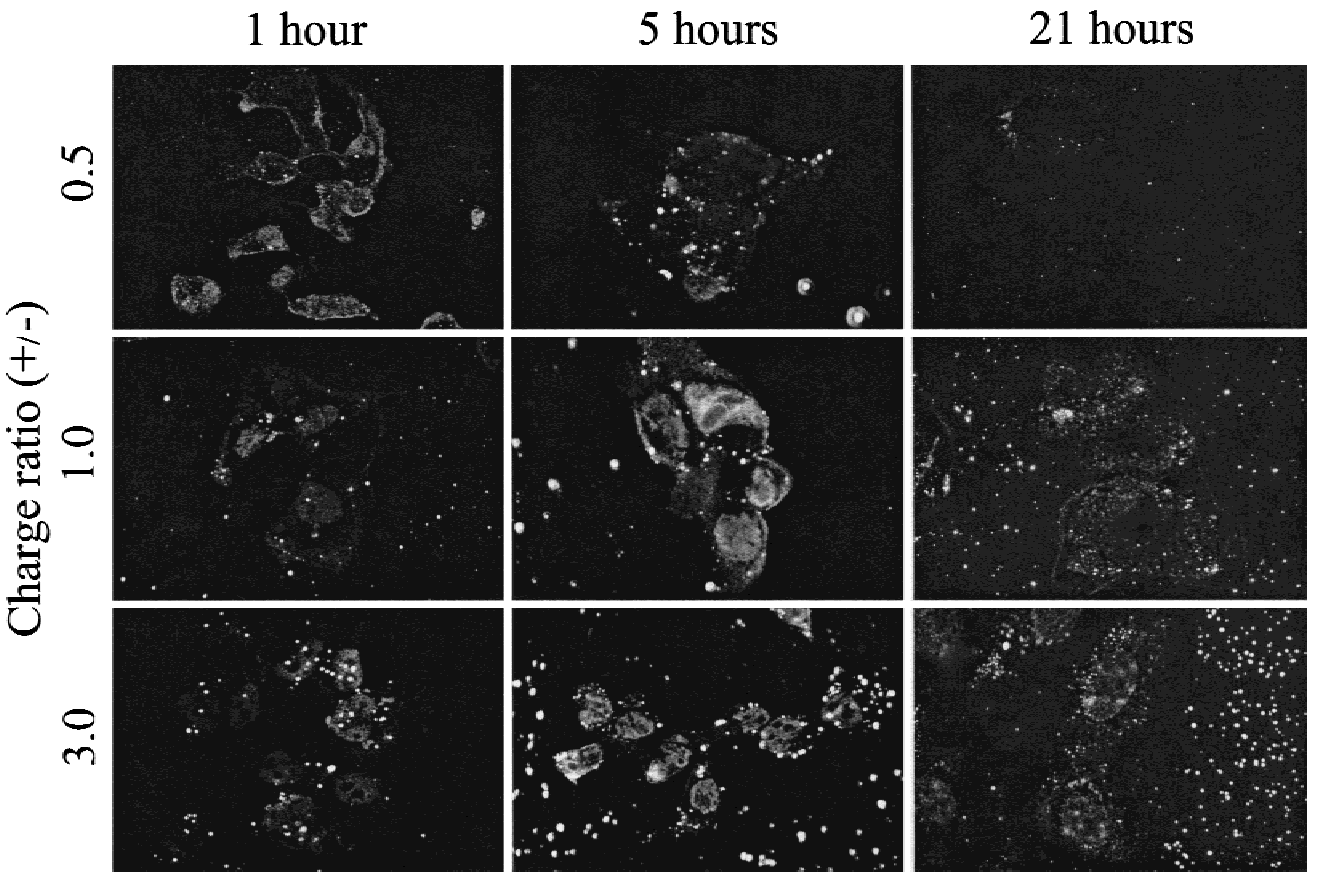

Fig. 3. Cellular uptake and fate of protamine sulfate-complexed DNA–DNA oligonucleotides with a charge ratio of 0.5 (+/–) (a, b, c), 1.0 (+/–) (d, e, f), and 3.0 (+/–) (g, h, i) after 1 hour (a, d, g), 5 hours (b, e, h) and 21 hours (c, f, i) of incubation. Contrast of the images was enhanced using contrast stretch in Confocal Assistant 4.02 software.

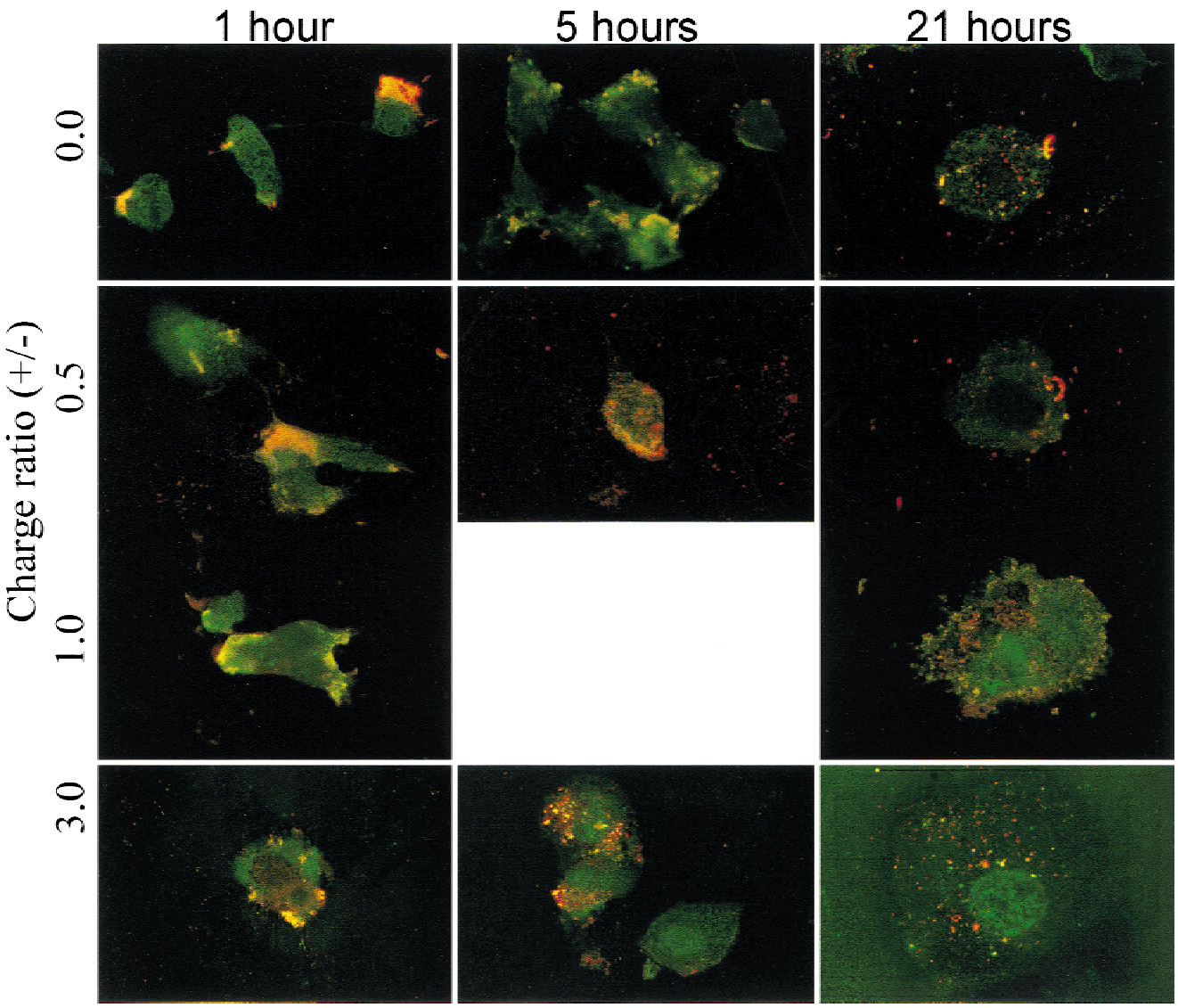

Fig. 4. Uptake and cellular fate of protamine sulfate-complexed DNA–DNA–ON after administration with AVE™-3 for different time points [1 hour: (a, d, g, i); 5 hours (b, e, j) and 21 hours (c, f, h, k)] and charge ratios [without protamine (a, b, c); 0.5:1 (+/–) (d, e, f); 1:1 (+/–) (g, h), and 3:1 (+/–) (i, j, k)]. Contrast of the images was enhanced using contrast stretch in Confocal Assistant 4.02 software.

that are coated with artificial viral envelopes (AVE™-3) is shown in Fig. 4. At a charge ratio of 3:1 (+/–) nuclear uptake of ON increased with incubation times. After 1 h, nuclear fluorescence is relative weak (Fig. 4 i), whereas after 5 and 21 hours incubation green fluorescence of ON accumulates selectively in the nucleus (Fig. 4 j and k). Lowering the PS:ON charge ratio to 1 (neutral complexes, Fig. 4 g and h) or to 0.5:1 (+/–) (Fig. 4 d–f) prior to coating with AVE™-3 is associated with decreased nuclear uptake which can be explained with an increase in nuclear elimination over time. Control experiments without PS present resulted in weak and transient nuclear fluorescence (Fig. 4 a–c).

Bright green colored spots—as could be observed with PS–ON complexes (without AVE™-3)—are not apparent on collagen-coated coverslips when PS–ON complexes are prepared with AVE™-3. Furthermore intracellular particulate green or yellowish fluorescent spots are associated with cationic charged complexes of PS with ON (Fig. 4 i–k).

Fluorescence intensity of confocal images of PS–ON–AVE complexes were quantified (Fig. 5). After a short-time incubation of 1 hour, a fluorescence ratio <1 for all charge ratios investigated indicates a higher concentration of ON in the cytoplasm. Increasing the incubation time to 5 hours, the nuclear fluorescence increased and after 10 hours fluorescence ratios peaked in all but one of the charge ratios (Fig. 5). Fluorescence ratio starts to decrease after 21 hours of incubation indicating nuclear elimination or degradation of fluorescent ON or fluorescence label. A maximum of fluorescence ratio is observed after 10 hours for a charge ratio of 3 (+/–), and after 21 hours of incubation with an excess of cationic charge.

With neutral liposomes instead of AVE™-3, there was no association of liposomes and oligonucleotides (data not shown).

DISCUSSION

A lack of sufficient cellular and nuclear uptake of naked ON has been reported by several investigators (6,20). The

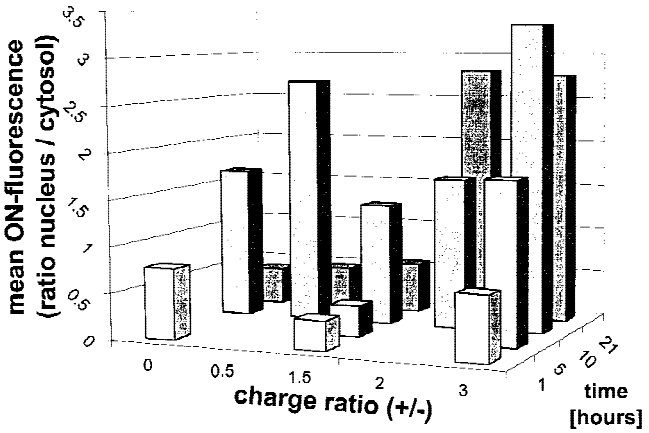

Fig. 5. Intracellular distribution kinetics of oligonucleotide (ON) fluorescence after transfection with AVE™-protamine sulfate-ON-complexes. The fluorescence ratio was calculated as quotient of mean ON-fluorescence intensity of nuclear and cytoplasmic fluorescence after background subtraction. Intraliposomal fluorescence of cytoplasmic distributed liposome associated ON-fluorescence was subtracted from total cytoplasmic fluorescence (see formula in Appendix). Analysis was performed with TIF-converted pic-files using Confocal Assistent 4.02 without any manipulation of raw data.

exact mechanisms of uptake are not clear. Receptor-mediated uptake (21), nonreceptor mediated uptake (22), and pinocytotic mechanisms (23) are being discussed.

Presumably due to their high negative charge, naked ON are poorly taken up by HepG2 cells. A weak cytoplasmic fluorescence after 1 hour is eliminated after 21 hours probably by enzymatic degradation of the ON and elimination of fluorescent label.

PS proves to effectively enhance the transport of ON into the nucleus of HepG2-cells. It is conceivable that enzymatic degradation of ON as it traverses the cytoplasm to the nucleus might still be a problem which can arise from the biological instability of pure PS–DNA complexes. A maximum of nuclear fluorescence was reached after 5 hours, and thereafter fluorescence in the nucleus starts to decrease. The ON is presumably degraded by intracellular nucleases, and the fluorescent label is eliminated from the cell or inactivated by intracellular metabolic processes. With cationic charge in excess, ON are presumably protected from enzymatic degradation in the cytoplasm and therefore sufficient ON entered the nucleus. Long-term stability of ON within the nucleus increases the chance of interaction with genomic DNA.

AVE™-3 associates with PS-condensed ON and with ON itself. Successful transfection with AVE™ and PS-condensed plasmid was demonstrated in HepG2-cells in the presence of serum (24). AVE™-3 has properties that enable passive nuclear targeting of ON. Using this liposomal formulation, ON could be delivered to the nucleus of cells. Cellular uptake of AVE™-3 coated PS–ON complexes is a result of co-transport to the cytoplasm as indicated by the yellow color which is a mixture of green ON-fluorescence and red AVE™-3-fluorescence. After 1 hour, ON are not released from the complexes, whereas after 5 hours green ON-fluorescence and red AVE™-3-fluorescence are distinguishable. A maximum of nuclear ON-fluorescence for the AVE–PS–ON complexes is observed after 10 hours for a 3:1 charge ratio (+/−). Decrease of nuclear ON fluorescence after 21 hours can be explained with enzymatic degradation of the unmodified ON or nuclear elimination of hydrolyzed fluorescence label. Co-uptake of AVE™-3 and PS–ON complex is important for successful development of modified AVE™-3 with targeting ligands to improve efficiency of the carrier system. PS–ON complexes are released from the liposomal formulation, mainly by enzymatic degradation of the natural phospholipids of the liposomal formulation. Punctuate structures within the cytoplasm may be endosomes containing PS–ON–AVE™-3 or release products of the complex. A liposome formulation similar to the HIV-1-envelope has been shown to be localized close to the nucleus whereas control liposomes composed of phosphatidylcholine and cholesterol are localized in the vicinity of the cytoplasm membrane (25). Localization near the nucleus of cytoplasmic ON-containing vesicles was also observed, especially with an excess of cationic charge and long-time incubation of 21 hours. Furthermore, AVE™ have fusogenic properties. For AVE™ similar to HIV-1-envelope, plain lipid envelope without CD4+-specific gp160 glycoprotein showed enhanced release of encapsulated FITC-dextran which is explained by its fusogenic properties due to the virus-like lipid composition (7).

Negatively charged AVE™-3 interact with cationic PS–ON complexes by charge attraction. AVE™-3 coating of PS–ON complexes prevents sticking of PS–ON complexes on collagen-treated coverslips. Using AVE™-3, bright green or yellow spots were only observed in cellular compartments (endosomes) with internalized PS–ON and AVE–PS–ON complexes, whereas after application of pure cationic PS–ON complexes, these are detectable on the surface of the coverslips as stable bright fluorescent complexes.

As neutral liposomes can be taken up by the cells independently from the PS–ON complex, the co-uptake of AVE™-3 and PS–ON complex is driven by charge attraction of liposomes and cationic PS residues. PS serves as an electrostatic linker between both negatively charged counterparts.

The lipids of AVE™-3 are enzymatically degradable, and fluorescence label may be metabolized or can be excreted from cells. However, it is rather likely that the lipids are not localized in the nucleus because nuclear localization of red fluorescence label is very weak, indicating that nuclear uptake of lipids is only taking place to a limited extent. A few observations of strong nuclear red-fluorescence (data not shown) correspond with observations by Godbey *et al.* (26) who showed cytoplasmic uptake of PEI–plasmid-complexes after 2.5 hours and nuclear uptake of these complexes after 3.5 hours. A nuclear localization of labelled cationic carrier system and labelled plasmid was evident. The mechanism of these observations is still not clear. One may speculate that the nuclear membrane may be transiently disturbed due to interaction with this synthetic cationic carrier system.

Our results indicate an extranuclear metabolism of lipids. The theory of disrupture of nuclear membrane by cationic protamine sulfate cannot be completely rejected up to now. However, the administration of protamine to patients to counteract heparin effects, or as an adjuvant to prolong insulin action in diabetic patients, is not associated with toxic effects.

The results of the kinetic analysis can be used to optimize transfection protocols for HepG2 cells with oligonucleotides in cell culture. Cellular uptake with our carrier system is com-

pleted after 3 hours and ON are present in the nucleus after 5 hours; therefore washing steps should be performed after 3–5 hours to remove adsorbed complexes that may be harmful to cells in culture. Therapeutic efficacy with respect to interaction with genomic DNA in the nucleus can be expected after 10 hours when the maximum of nuclear fluorescence is reached.

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APPENDIX

Formula for Calculation of Fluorescence Ratios (see Fig. 5)

$$ratio_{oligo}\frac{n}{c}=\frac{mFL_{oligo}(n)-mFL_{oligo}(b)}{\frac{A(c+l)*mFL_{oligo}(c+l)-A(l)\,mFL_{oligo}(l)}{A(c)}-mFL_{oligo}(b)}$$

Where $mFL_{oligo}(b,c+l,l,c,n)$ = mean ON fluorescence, $A(c+l,c)$ = integrated area, b = background, $c+l$ = cytosol + liposomes, l = liposomes, c = cytosol, and n = nucleus.