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Biochimica et Biophysica Acta 1663 (2004) 127–134

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Transfection efficiency and cytotoxicity of cationic liposomes in salmonid cell lines of hepatocyte and macrophage origin

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Received 1 July 2003; received in revised form 20 January 2004; accepted 27 February 2004

Available online 19 March 2004

Abstract

The transfection efficiency of liposome-based DNA formulations was studied in different salmonid cell lines of hepatocyte and macrophage origin. Parallel assessment of cell viability was carried out to define the balance between transfection efficiency and toxicity. For all cell lines, transfection efficiency varied with the lipoplex charge ratio and the amount of DNA added to the liposomes. The hepatocyte-derived cell line was most readily transfected while lower transfection efficiency was observed for the macrophage cell lines. The cationic liposomes showed a dose-dependent toxicity and were found to be most toxic for cells of macrophage origin. This was in line with the observation that higher amounts of lipids were associated with the cells of macrophage origin than the hepatocytes. Complexing DNA with the liposomes reduced the toxicity for all three cell lines, most markedly, however, for macrophage cell lines. The differences in the transfection and toxicity patterns between the cell lines are probably caused by differences in membrane composition as well as differences in phagocytic activity and processing of the liposomes/lipoplexes.
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Keywords: Transfection; Toxicity; Macrophage; Hepatocyte; Cationic liposome; DNA

1. Introduction

Well-designed delivery systems are designed to increase the efficiency of plasmid delivery and a widely used delivery system in mammals is cationic liposomes [1]. Cationic liposomes have also been employed for the delivery of plasmids to fish [2–4]; however, a general disadvantage of this system is high toxicity to cells [5–7]. This must be taken into consideration when determining the optimal transfection conditions and the key is to balance the toxicity of the lipid and the level and efficiency of transfection. In vitro transfection efficiency and toxicity of cationic liposomes have been characterised in a variety of mammalian cell types [8–11]. In fish, however, such studies are more limited and have been performed in primary cultures of gill cells [12] and in the *Epithelioma papulosum cyprini* (EPC) cell line [12,13].

Macrophages play an important role in the uptake and clearance of both soluble and particulate antigens in fish, e.g. liposomes [14], latex particles [15], injected bacteria [16], and vaccine components [17]. The spleen and the head kidney are the major centres of the mononuclear phagocyte system in the rainbow trout and these secondary lymphoid organs of fish are of crucial importance for immune induction [18]. Also the peritoneal cavity contains macrophages that rapidly phagocytose injected bacteria [19]. Following intraperitoneal injection of a cationic liposome-formulated reporter gene to rainbow trout, expression was as expected detected in head kidney and spleen [2]. Surprisingly, expression was also detected in the liver, an organ with no known immunological function in salmonid fish.

Fish generally have a lower body temperature than mammals. At a cellular level, this is seen as a difference in the fluidity of the cell membranes, reflected by a different lipid composition of the membranes [20]. For this reason, the interactions between fish cells and the delivery system may be different from what is observed in mammalian systems. The aim of this study was to investigate the

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doi:10.1016/j.bbamem.2004.02.007

transfection efficiency and toxicity of a liposome-based DNA delivery system for salmonid cell lines of hepatocyte and macrophage origin.

2. Materials and methods

2.1. Chemicals

The plasmid construct pcDNA3-luc was obtained from GeneCare (Lyngby, Denmark). In this construct, the gene encoding luciferase was inserted down-stream of the immediate–early enhancer–promoter sequences of human cytomegalovirus in a pcDNA3 vector (Invitrogen, Groningen, the Netherlands). L-α-Dioleoylphosphatidyl-ethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and the fluorescence-labelled lipid *N*-(7-nitrobenz-2-oxz-1,3-diazol-4-yl)phosphatidylethanol-amine (NBD-PE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Leibowitz's L-15 medium with L-glutamine, gentamicin, penicillin G/streptomycin sulfate (PEST) and trypsin were obtained from GibcoBRL ® (Invitrogen). Fetal bovine serum was purchased from BioWhittaker (Verviers, Belgium). The luciferase assay system and the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) were from Promega Corporation (Madison, WI, USA) while the Bio-Rad Protein Assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents were of analytical grade.

2.2. Preparation and characterisation of the liposome and lipoplex formulations

The liposomes were prepared according to the classical film hydration method. Briefly, thin films were obtained by rotary evaporation of chloroform solutions of DOPE/DOTAP 1:1 (liposomes) and DOPE/DOTAP/NBD-PE 1:1:0.04 (NBD-liposomes), both ratios are weight/weight ratios. The lipid films were kept under vacuum for an additional 15 min to remove residues of organic solvents. Thereafter, glass beads were added and the films were hydrated in distilled, de-ionised water. After 2 h, the liposome suspensions were extruded 10 times through two-stacked polycarbonate membranes (100 nm) with a Lipex extruder (Lipex Biomembranes, Vancouver, Canada).

The concentration of the liposomes was determined according to the Rouser assay [21]. Briefly, liposome and calibration curve samples were evaporated to dryness in a block heater. Thereafter, 0.3 ml perchloric acid was added, the tubes were covered with marbles and kept in the block heater (160 °C) for 120 min. One milliliter distilled, de-ionised water, 0.4 ml 1.2% hexa-ammoniummolybdate solution and 0.4 ml 5% ascorbic acid was added when the samples were cooled down, the samples were mixed and kept at 100 °C for 5 min (water bath). The absorbance at 797 nm was read in a UV spectrophotometer and the amount of phosphate in the samples was calculated from the calibration curve. The relative standard deviations of the liposome concentrations were $\leq 1\%$ when measured by the Rouser assay.

To form lipoplexes, equal volumes of DOPE/DOTAP 1:1 and plasmid (both diluted to appropriate concentrations) were mixed, resulting in charge ratios of 0.5, 1, 1.5, 2, 3 and 5. The lipoplexes were kept at room temperature for at least 30 min before use.

The size and zeta potential of liposomes (500 μg/ml) and lipoplexes (DNA concentration 8 μg/ml), diluted 1:3 in serum-free cell medias, were measured at 25 °C by dynamic light scattering (Zetasizer 1000HSA, Malvern Instruments, GB) and by micro-electrophoresis (Zetasizer 2000, Malvern Instruments, GB), respectively.

2.3. Cells

Three different cell lines originating from salmonid fish were used in this study. The RTH-149 cell line originates from an aflatoxin-induced hepatoma in an adult rainbow trout [22] and has retained some characteristics of normal hepatocytes [23]. The RTH-149 cells, obtained from ATCC (Rockville, MD, USA), were cultured at 20 °C in L-15 medium supplemented with 10 mM glutamic acid, 10 mM $NaCO_3$, 50 μg/ml gentamicin and 5% FBS. The macrophage-like cell line RTS-11 is developed from rainbow trout spleen [24]. RTS-11 cells were cultured at 20 °C in L-15 medium supplemented with 150 U/ml penicillin G, 150 μg/ml streptomycin sulfate and 25% FBS and were maintained as described by Brubacher et al. [25]. The cell line SHK-1, developed from Atlantic salmon head kidney leucocytes, also show macrophage-like characteristics [26]. The SHK-1 cells were cultured in L-15 supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 5% FBS. The RTH-149 cell line is referred to as hepatocytes or cell line of hepatocyte origin, while the two latter cell lines are termed macrophages or cell lines of macrophage origin.

2.4. Transfection experiments

2.4.1. Transfection with pcDNA3-luc

The cells were seeded at a concentration of 500,000 cells/well in a 24-well plate 24 h prior to the transfection. The serum-containing medium was then removed and replaced with 750 μl serum-free medium and 250 μl of the formulations. After 24 h incubation at 20 °C, the serum-free medium and the formulations were removed and replaced with 1 ml complete medium. The cells were lysed in a lysating buffer (25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100®) after a further 2 days and the lysates were assayed for luciferase according to the manufacturers description using a Lumat LB 9507 Luminometer (EG&G Berthold, Germany). The relative light units (RLU) were normalised to the protein concentration as determined by the Bio-Rad assay.

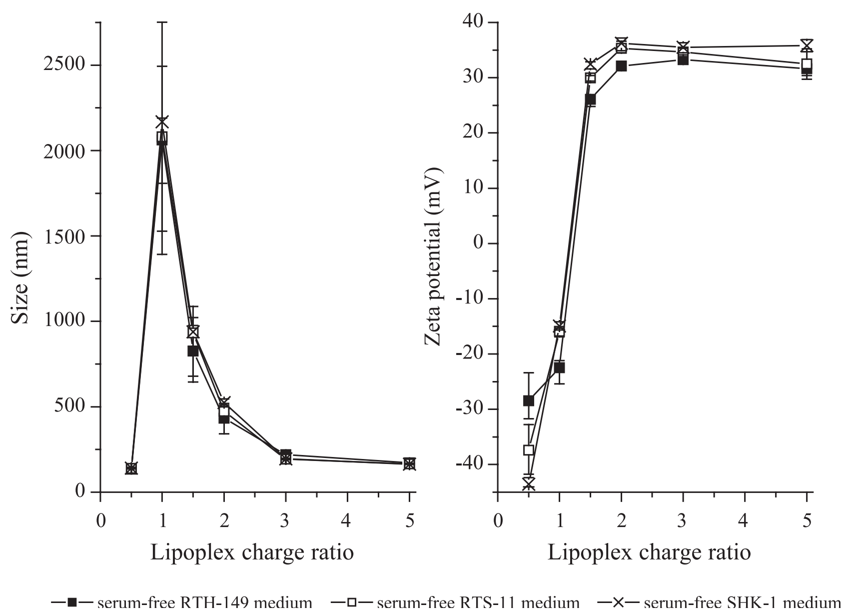

Fig. 1. The size and zeta potential of lipoplexes diluted 1:3 in the three serum-free cell media. The results are given as the mean ± max/min values ($n=3-4$).

2.5. Viability studies

The cells were seeded at a concentration of 100,000 cells/well in a 96-well plate. After 24 h, the medium was removed, and 50 μl serum-free and 50 μl liposome/lipoplex-formulations were added. The liposomes/lipoplex and the serum-free medium were removed after 24 h at 20 °C and 100 μl serum-containing medium was added to each well. After another 24 h, 20 μl MTS was added per well, and the absorbance was read at 490 nm the following day.

2.6. Association of the cationic liposomes with the cells

The cells were seeded at a concentration of 500,000 cells/well in a 24-well plate 24 h prior to the start of the experiment. The serum-containing medium was then removed, the cells were washed twice with PBS and 750 μl serum-free medium and 250 μl of the NBD-liposomes was added per well. After 24 h at 20 °C the serum-free medium and the NBD-liposomes were removed by washing the cells twice with PBS, after which 200 μl PBS containing 1% Triton X-100® was added to the cells. The fluorescence was measured using the excitation and emission wavelengths at 460 and 535 nm, respectively (Luminiscence Spectrofotometer LS50B (Perkin-Elmer, Bodenseewerk, Germany). The results were expressed as micrograms of associated lipid per 500,000 cells. Differences in associated lipids between the three cell lines were analysed with Student's *t*-test.

3. Results

3.1. Characterisation of the liposome and lipoplex formulations

The size and the zeta potential of the lipoplexes diluted in the serum-free cell-media RTH-149, RTS-11 and SHK-1 are shown in Fig. 1. The particle size was dependent on the lipoplex charge ratio. The smallest particles were observed for lipoplex 0.5 (135–140 nm). A maximum size was reached at a charge ratio of 1 (lipoplex 1), followed by a size reduction at higher ratios. There were no marked differences in the size of the lipoplexes in the different cell media; the liposomes, however, were of a larger size in the serum-free RTH-149 medium (Table 1). The zeta potential of the lipoplexes was also dependent on the charge ratio. A shift in the zeta potential, indicating a change from an overall negative to a positive charge of the lipoplexes, occurred between lipoplex 1 and lipoplex 1.5. The zeta

Table 1
The size and zeta potential of the liposomes diluted 1:3 in the three serum-free cell media

Formulation	Size (nm)			Zeta potential (mV)		
	RTH-149	RTS-11	SHK-1	RTH-149	RTS-11	SHK-1
Liposomes	163 ± 4.5	85 ± 2.3	90 ± 1.6	26.1 ± 0.83	38.7 ± 2.84	38.2 ± 2.08
NBD-liposomes	164 ± 1.4	100 ± 2.7	100 ± 1.2	26.4 ± 1.76	39.2 ± 2.63	37.8 ± 1.80

The results are given as the mean ± S.D. ($n=3-4$).

potential and the size of the NBD-liposomes were similar to the unlabelled liposomes (Table 1). The pH of the different cell media were 8.4 (serum-free RTH-149 medium) and 7.6 (serum-free RTS-11 and SHK-1 media).

3.2. Transfection experiments

The expression of luciferase following transfection with lipoplexes of different charge ratios in the RTH-149, RTS-11 and the SHK-1 cell lines is shown in Fig. 2. The highest levels of luciferase expression were detected in the RTH-149 cells, these values were approximately 2 log-units higher than for the macrophage cell lines for all formulations tested. The highest levels of luciferase expression was detected following transfection with lipoplex 1 and lipoplex 1.5 in the RTH-149 cells (average log RLU/mg protein = 7.6). In the RTS-11 cells, the highest luciferase expression was also detected following transfection with lipoplex 1 (5.8), while in the SHK-1 cell line, lipoplex 5 gave the highest average luciferase value (4.8). This value, however, was not significantly higher than what was obtained for lipoplex 1.5 or lipoplex 2.

The expression of luciferase following transfection with lipoplex 1 at increasing DNA concentrations is shown in Fig. 3. Again, the highest levels of luciferase expression were detected in the RTH-149 cells. Luciferase activity was detected already at 0.5 μg DNA per well (average log RLU/mg protein = 7.1) and the average values increased to 7.7 using 10 μg DNA. One microgram of DNA per well was required to obtain detectable levels of luciferase in the RTS-11 cells. The highest level of transfection in this cell line, however, was obtained with 2 μg DNA per well (average log RLU/mg protein = 5.8). At 10 μg DNA per well, the level of luciferase expression was lower and at 15 μg DNA/well, no luciferase expression could be detected (not shown). Detectable levels of luciferase were measured in the SHK-1 cells at 2 μg DNA per well. Increasing the amount of DNA to 5 and 10 μg/well resulted in increasing levels of luciferase expression in the SHK-1 cells (average log RLU/mg protein = 5.4).

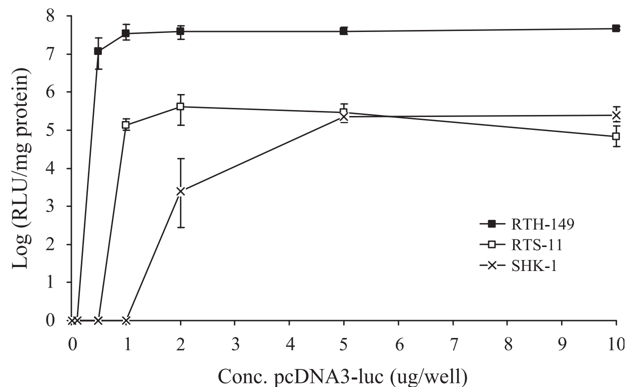

Fig. 3. The expression of luciferase in the RTH-149, RTS-11 and SHK-1 cell lines following transfection with lipoplex 1 and various concentrations of pcDNA3-luc. The results are given as the mean ± max/min values ($n = 3$).

3.3. Viability studies

The relative viability of the three cell types after exposure to different concentrations of DOPE/DOTAP 1:1 liposomes is illustrated in Fig. 4. The liposomes were toxic for the macrophage-like cell lines, RTS-11 and SHK-1, and the viability of the cells decreased by 30% and 35%, respectively, when the liposome concentration increased from 0 to 20 μg/ml. By contrast, the reduction in viability was only 5% in the RTH-149 cells over the same range. A further increase in liposome concentration decreased the viability of all cell lines. The viability of the RTS-11 and SHK-1 cells was below 15% for concentrations above 100 μg/ml liposomes. At 100 μg/ml, the viability of the RTH-149 cells was still 84%, while around 30% of the cells remained viable at liposome concentrations >500 μg/ml.

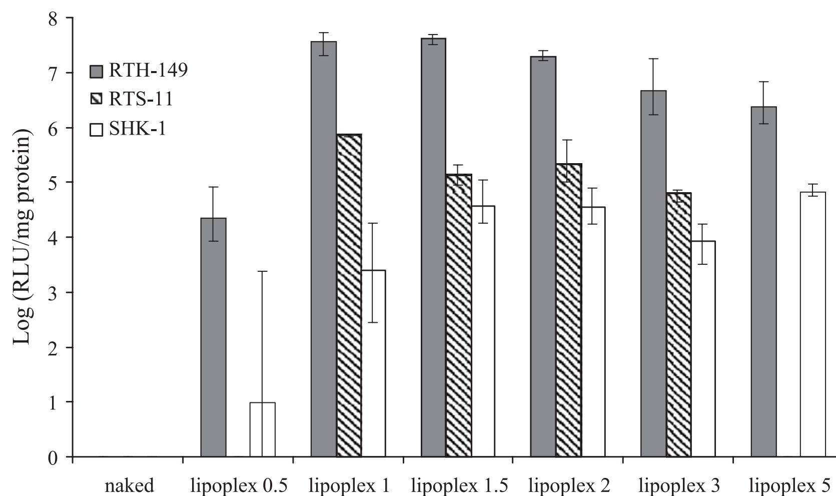

Fig. 2. The expression of luciferase in the RTH-149, RTS-11 and SHK-1 cell lines following transfection with lipoplexes of different charge ratios. pcDNA3-luc (2 μg) was used per well and the results are given as the mean ± max/min values ($n = 3$).

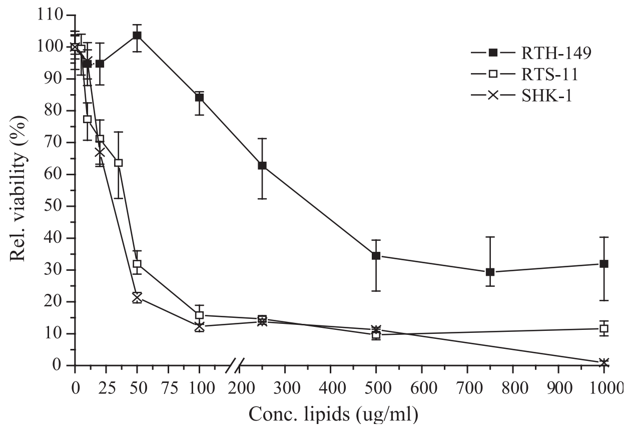

Fig. 4. The cytotoxicity of liposomes (DOPE/DOTAP 1:1) on the RTH-149, RTS-11 and SHK-1 cell lines. The values are expressed as mean ± max/min values (n = 4–5).

The toxicity was reduced when liposomes were added to the cells in formulation with DNA (Fig. 5). The viability of the RTS-11 and SHK-1 cells was influenced by the amount of DNA in the lipoplexes (lipoplex 0.5–lipoplex 5) and there was a gradual increase in viability when the DNA concentration increased from 2.4 to 24 μg/ml (at lipid concentrations of 50 μg/ml) or 11.8 to 118 μg/ml (for lipid concentrations of 250 μg/ml). The concentration of lipids in the lipoplexes was also of importance, and the number of cells surviving was higher at a concentration of 50 μg/ml lipids compared to 250 μg/ml. Concordant with previous findings, the viability of the RTH-149 cells was not affected when exposed to liposomes/lipoplexes made with a lipid concentration of 50 μg/ml (Fig. 5A). At a lipid concentration of 250 μg/ml, lipoplexes with charge ratios of 2, 1 and 0.5, gave similar results in RTH-149 cells, while the viability was reduced for lipoplex 5 exposure at this lipid concentration (Fig. 5B).

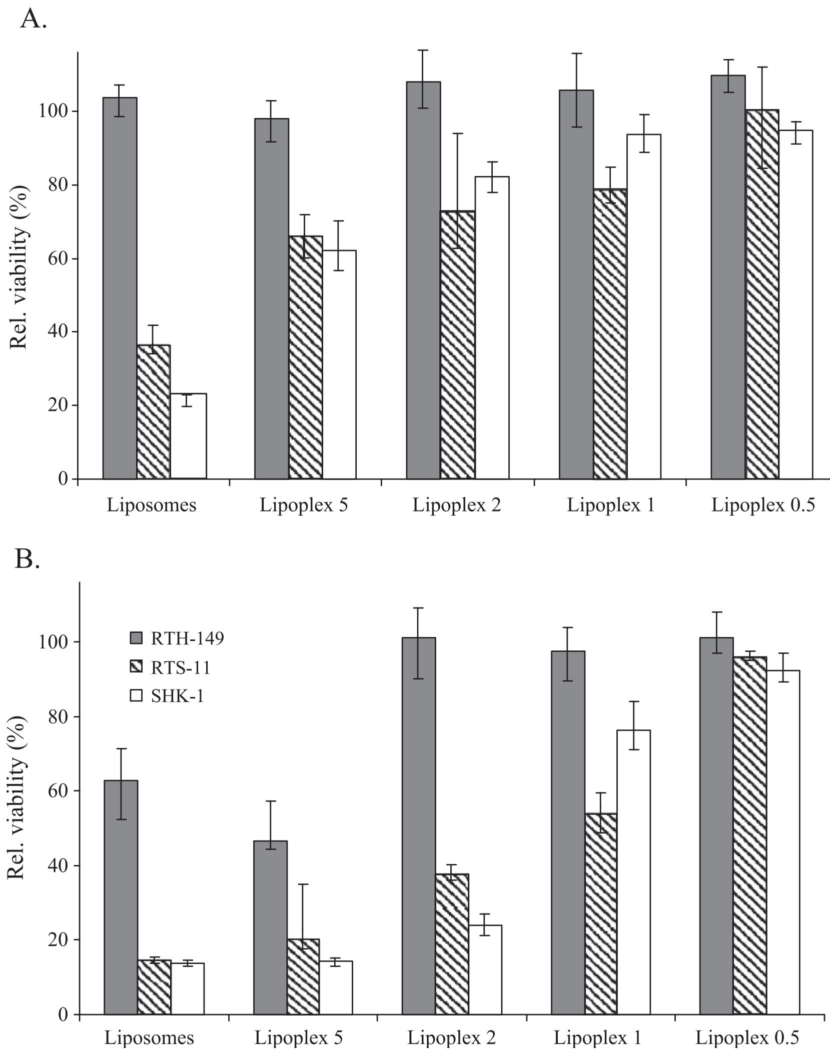

Fig. 5. The cytotoxicity of lipoplex on the RTH-149, RTS-11 and SHK-1 cell lines. The concentration of the lipids was 50 μg/ml (A) or 250 μg/ml (B). The values are expressed as mean ± max/min values (n = 4–5).

Table 2
The association of fluorescence-labelled DOPE/DOTAP liposomes (NBD-liposomes) with RTH-149, RTS-11 and SHK-1 cell lines

Cell line	Associated lipid (μg)/500,000 cells
RTH-149	5.3 ± 0.23
RTS-11	11.7 ± 0.96
SHK-1	7.2 ± 1.04

The concentration of the lipids during exposure was 250 μg/ml. The results are given as the mean ± S.D. ($n = 3$).
P-values: $P = 0.004$ (RTS-11 vs. RTH-149), $P = 0.005$ (RTS-11 vs. SHK-1), $P = 0.035$ (SHK-1 vs. RTH-149).

3.4. Association of the cationic liposomes with the cells

The highest association of NBD-liposomes with the cells was detected in the macrophage RTS-11 cell line (11.7 μg lipid/500,000 cells), followed by the macrophage SHK-1 cell line (7.2 μg lipid/500,000 cells) (Table 2). The association of NBD-liposomes with the hepatocyte cell line RTH-149 was significantly lower, 5.3 μg lipid/500,000 cells.

4. Discussion

There are number of steps and barriers that must be passed in order to obtain a successful transfection [27]. The first step is the formation of the lipoplex, in which the plasmid is bound electrostatically to the liposome. Thereafter, the lipoplex must interact and associate with the cell membrane followed by an uptake of the lipoplex into the cells. The lead theory claims that the mechanism of uptake is endocytosis, although direct fusion with the cell membrane is also mentioned as a possible mechanism [27–30]. Once internalised, the intracellular vesicles carrying the vectors fuse with organelles collectively referred to as the endocytic compartment. Endosomal escape is necessary for efficient gene delivery. The mechanism of escape is thought to involve lipid mixing between the endosomic and cationic lipid membranes, which leads to disruption and release of DNA into the cytoplasm. However, the exact mechanism is not defined and can vary between different cells and lipid delivery systems [30,31]. In the end, DNA must enter the nucleus and it is believed that DNA could either pass into the nucleus through nuclear pores, become physically associated with chromatin during mitosis when the nuclear envelope breaks down, or it could transverse the nuclear envelope [27,30]. In this paper, we have compared fish cell lines of hepatocyte and macrophage origin with regard to transfection efficiency for a liposomal delivery system. In addition, we have aimed at characterising the adsorption/internalisation step in more detail and correlate this with transfection efficiency.

The transfection experiments showed that the hepatocytes were more easily transfected than the cell lines of macrophage origin. In mammals, gene transfection is known to be cell type-dependent and macrophages are known to be difficult to transfect with a liposomal delivery system [10]. This is concordant with what we have found as fish cell lines of a macrophage origin are difficult to transfect with a liposomal delivery system. There may be several reasons for this. Firstly, one may argue that a liposomal delivery system is not the optimal gene delivery system for macrophages. However, since the transfection efficiency is low also with other gene delivery systems in macrophages [32], it is reasonable to assume that the delivery system itself is not the only cause of low transfection efficiency. Contamination of plasmid DNA preparations with endotoxin may be of importance, as it has been shown that endotoxin severely limited gene expression in macrophages but had little or no effect in other cell lines [33]. The endotoxin content in the plasmid solution used in this study was 72 EU/ml, which may have influenced the transfection efficiency in the macrophage cells lines. Other mechanisms must also be taken into account. Macrophages are professional phagocytes and will easily take up liposome particles by phagocytosis. Phagocytosed material will normally be degraded through the lysosomal route and a reduced endosomal escape of the liposomes will possibly result in low transfection efficiency. Further studies should be pursued to explore these mechanisms in more detail.

The transfection efficiency was dependent on the amount of DNA and the lipoplex charge ratio for all cell lines. These results are in agreement with studies performed in mammalian cells, where these factors were found to be of importance in both macrophages and primary cultures of hepatocytes [9,10,34]. A net positive charge of the lipoplex is considered important to allow electrostatic interactions with the overall negative charge of the target cell membrane [35]. In addition, the size of the lipoplexes may play a role because large particles sediment and possibly facilitate cell contact [27,36]. In our studies, lipoplex 1 and 1.5 generally gave the highest levels of transfection. The charge of the lipoplexes shifted from a net negative to a net positive between lipoplex 1 and 1.5, these lipoplexes also had the largest particle size. Expression of luciferase after intraperitonal injection of lipoplexes has been detected in the spleen, head kidney and liver of rainbow trout [2] and the values obtained for lipoplex 2 were higher than for lipoplex 5. Like for the in vitro studies, this may be caused by differences in lipoplex size. On the other hand, also other factors are likely to influence, e.g. the charge, lipid content and structure of the lipoplexes.

The cytotoxicity of the cationic liposomes increased with increasing lipid concentration for all three cell lines. Cationic lipids are known to be membrane active [37]. They may therefore interfere with the membrane function and integrity of the cell or the subcellular compartments and lead to toxicity. Also, the presence of the pH-sensitive lipid DOPE in the liposomes may contribute to the toxicity. The success of transfection is determined by the intracellular fate following uptake into the cells; most of the complexes are degraded in the lysosomes. Due to the formation of an

inverted hexagonal phase at acidic pH, a pH typically found in the lysosomes, DOPE may enhance the cationic lipid toxicity by destabilising the lysosomal membrane [7].

The toxicity to the three fish cell lines was reduced following addition of DNA to the liposome formulations which may be explained by the shift in zeta potential of the complexes. This is also observed in other types of fish cells [12]; however, it is in contrast to studies by Filion and Phillips [7] where it was shown that incorporation of DNA in the liposomes only marginally reduced the toxicity to macrophages. The discrepancy observed may be due to different experimental conditions as well as differences between fish cells and mammalian cells.

Fluorescence-labelled liposomes were used to study in more detail the initial association of the liposome-formulations with the cell. The association of lipids to the macrophages was higher than for the hepatocytes but the transfection rate was higher in the hepatocytes. First of all, the lack of correlation between a high degree of association of liposomes with transfection efficiency in macrophages is comparable to what has been observed in mammalian macrophage cell lines [8]. Secondly, it is reasonable to assume that the properties or components of the medium can influence the adhesion of liposomes to the cells and pH of the medium was found to influence on the size and zeta potential of the liposomes. The low association of liposomes with RTH-149 cells may have been influenced, directly and indirectly, by the higher pH of the medium used for this cell type, but this is not reflected in transfection rate.

In mammalian cells, it is possible to distinguish between internalised and adsorbed liposomes by carrying out studies at 4 and 37 °C [7,8]. This is not so easy for fish cell lines as complete inhibition of macrophage activity in fish cells cannot be obtained at 4 °C [38]. Use of metabolic inhibitors (NaN_2 and cytochalasin B) gave cytotoxic effect in the cell cultures (even at very low concentration) and attempts to visualise adhesion/internalisation by confocal fluorescent microscopy were unsuccessful. Thus, it has been shown that association of fluorescent liposomes is highest in the macrophage cell lines but transfection efficiency is significantly higher in the hepatocyte cell lines. The underlying mechanisms cannot be explained in detail.

The observed differences in toxicity in macrophage cells lines compared to hepatocytic ones may be caused by a higher adsorption of the cationic liposomes with the macrophage cell membranes. This may be due to variations in molecular structure and surface charge of macrophages vs. the hepatocytes, characteristics that may reflect a variability of the cell proteoglycans [35]. Another explanation for the cell-variable toxicity may be the high phagocytic capacity of the macrophages. As has been shown in mammalian systems, cationic liposomes are toxic for phagocytic macrophages and monocyte-like cells [7], but not so for non-phagocytic T lymphocytes [7] or primary hepatocytes of mammalian origin [9]. An additional possibility is the induction of apoptosis by cationic liposomes, which in mammalian systems is shown in macrophages but not in hepatocytes [39].

In conclusion, the differences in transfection efficiency and cytotoxicity between the hepatocyte and macrophage cell lines are most likely explained by variations in membrane composition as well as phagocytic ability and subsequent processing of the liposomes/lipoplexes. Further studies should be pursued to understand these phenomena in more detail, possible also with comparative studies in warm-blooded animals.

Acknowledgements

This work was supported by the Inger R. Haldorsen Fund, Alpharma AS and EC FAIR CT-98-4087.

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