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Gene delivery by negatively charged ternary complexes of DNA, cationic liposomes and transferrin or fusigenic peptides

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RESEARCH ARTICLE

Increased receptor-mediated gene delivery to the liver by protamine-enhanced-asialofetuin-lipoplexes

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A novel lipidic vector composed of DOTAP/Chol liposomes, asialofetuin (AF), protamine sulfate and DNA has been developed. The resulting protamine-AF-lipoplexes improved significantly the levels of gene expression in cultured cells and in the liver upon i.v. administration. Lipoplexes containing the optimal amount of AF (1 μg/μg DNA) showed a 16-fold higher transfection activity in HepG2 cells than non-targeted (plain) complexes. The uptake by cells having asialoglycoprotein receptors (ASGPr) on their plasma membrane was decreased by the addition of free AF, indicating that AF-lipoplexes were taken up specifically by cells via ASGPr-mediated endocytosis. Results from transfections performed in cells defective in ASGPr, ie HeLa cells, confirmed this mechanism. By addition of the condensing peptide, protamine sulfate, smaller complexes were obtained, which enhanced even more the uptake of AF-complexes in HepG2 cells and in the liver. The optimal amount of protamine was 0.4 μg/μg DNA, and gene expression was about 5-fold over that obtained with AF-lipoplexes in the absence of the peptide, and 75-fold higher than that with plain conventional lipoplexes. Protamine-AF-lipoplexes increased by a factor of 12 luciferase gene expression in the liver of mice administered systemically via the tail vein, compared to plain complexes. In summary, our findings extend the scope of previous studies where AF-lipoplexes were used to introduce DNA into hepatocytes. The combination of targeting and protamine condensation obviated the need for partial hepatectomy, commonly required to obtain efficient gene delivery in this organ. Since protamine sulfate has been proven to be non-toxic in humans, the novel liver-specific vector described here may be useful for the delivery of clinically important genes to this organ.
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Keywords: *cationic liposomes; asialoglycoprotein receptor; protamine sulfate; asialofetuin; hepatocyte; liver gene therapy*

Introduction

The liver possesses a variety of characteristics that make this organ very attractive for gene therapy. The proportion of administered macromolecules internalized by hepatocytes depends on their particle size and biochemical characteristics. Only relatively small molecules can pass the fenestrae of sinusoidal endothelial cells in the liver, since their diameter is about 100 nm. On the other hand, the basic mechanism underlying targeted delivery is ligand-receptor interactions. Specific targeting to the liver has been achieved by using ligands that bind the asialoglycoprotein receptor (ASGPr), which is uniquely present on hepatocytes in large numbers with high-affinity binding.[1] Asialofetuin (AF), a glycoprotein having triantennary galactose terminal sugar chains, is known as an excellent ligand molecule selectively recognized by ASGPr.[2]

For liver gene delivery, both virus-mediated[3–5] and non-viral systems have been considered. Although some of the virus-mediated gene transfer systems have been found to be quite effective, their usefulness is limited, given that they induce an immune response, leading to the rapid rejection of transduced cells. To overcome this problem, artificial, non-viral gene delivery systems are being developed, including small liposomes,[6–8] particle bombardment,[9] gene gun,[10] electroporation,[11] chylomicron remnants[12] and cationic polymers.[13] Successful transfer and expression in the liver can also be achieved by systemic administration of naked plasmid using a hydrodynamic-based procedure.[14,15] One major concern for this technique is that it requires an injection volume of 8% of the body weight, which may alter the physiological conditions of the liver. Localized hepatic gene expression has also been achieved using direct hepatic DNA injection.[16]

The feasibility of targeted gene delivery to hepatocytes through the ASGPr has been amply demonstrated by the work of Wu *et al*.[17–22] In this system, DNA molecules are complexed with a stretch of polylysine (PLL), which is coupled to the asialoglycoprotein moiety. PLL residues are intended to interact with DNA and the asialoglycoprotein behaves as a homing device to target hepatocytes. Although effective, these complexes have some limitations. The ratio of asialoglycoprotein to PLL is somewhat unpredictable, resulting in heterogeneous and unstable complexes, with different DNA-binding capacities. Another problem, apart from the low efficiency, is that the strong affinity of PLL for cells[23] limits the

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targeting ability of these conjugates despite the inclusion of cell-binding ligands. Furthermore, the activation of components of the complement system by PLL reduces the half-life of ligand-PLL/DNA complexes *in vivo*. Other authors have also designed different systems targeted to the ASGPr[24,25] in the presence of PLL.

Lipoplexes have become a widely used non-viral gene delivery system and are formed when cationic liposomes are mixed with plasmid DNA. Various liposomal formulations containing modified galactolipids have been optimized for maximum liver uptake.[26,27] Hara *et al*[28–30] reported that AF-labeled liposomes encapsulating plasmid DNA is effective in gene expression after intraportal injection with a preload of EDTA. On the other hand, Zanta *et al*[31] reported ASGPr-mediated *in vitro* gene delivery by a 5% galactose-bearing-PEI-polymer. The major limitation of most of these methods is that they require a local administration or surgical procedures. The resulting level is relatively low and restricted to the injection site, and sustained expression often implies previous partial hepatectomy.[19,21,32,33]

Protamine sulfate, as a component of non-viral vectors, may promote the delivery of DNA from the cytoplasm into the nucleus. It has been proposed that this peptide increases lipid-mediated gene transfection by condensing DNA into a compact structure, which promotes cellular entry and stability of DNA.[34,35] The use of this polycation could also improve the *in vivo* activity of lipoplexes[36,37] and has the additional advantage of being non-toxic in humans. In this study we examined whether a liver-targeted, stable gene transfer system could be constructed by combining the condensing effect of protamine and the targeting capability of AF.

Results

Optimization of asialofetuin-lipoplexes

In order to obtain efficient complexes for gene delivery *in vitro* and *in vivo*, lipoplexes were prepared in the presence of different amounts of AF. The particle size, zeta potential and the protection of the plasmid inside the complexes from attack by degrading enzymes *in vitro*, were determined as a function of the amount of the ligand. Figure 1 shows the values of the particle size and the zeta potential of AF-liposomes and lipoplexes at 4/1 (+/−) charge ratio containing plasmid DNA (1 μg of pCMVLuc) in the absence or presence of increasing amounts of AF. It is important to consider that the size and the overall charge of starting liposomes was 120 nm and 47 mV, respectively. By increasing the amount of asialofetuin to 1 μg (per microgram of DNA) the particle size of AF-liposomes increased slightly to approximately 130 nm. The same behavior was observed for AF-lipoplexes, which reached a size of 302 nm. The zeta potential of AF-liposomes and lipoplexes showed clearly positive values by using amounts of asialofetuin lower than 1 μg. AF-complexes aggregated at 4.5 and 9 μg AF/μg DNA, which corresponds to a value of the zeta potential close to the electroneutrality. AF-liposomes in the presence of 4.5 μg AF also aggregated, and exhibit a zeta potential close to zero. Amounts of the ligand above 18 μg lead to AF-liposomes and lipoplexes of 130 nm with a markedly negative surface charge.

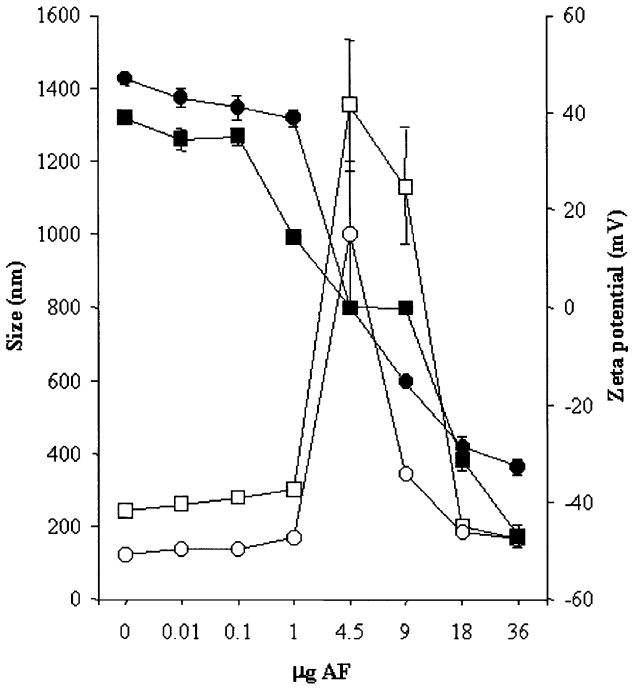

Figure 1 *Optimization of AF-lipoplexes. Particle size (open symbols) and zeta potential (closed symbols) of DOTAP/Chol liposomes (circles) and lipoplexes (squares) in the absence or presence of different amounts of asialofetuin. Each value represents the mean ± s.d. (n=3).*

To establish whether the plasmid inside the complexes is protected from degradation by DNases, an *in vitro* DNase I protection assay was performed. Lipoplexes were prepared at 4/1 (+/−) charge ratio and contained 2.5 μg of pCMVLuc. Gel electrophoresis data indicated that naked DNA was degraded quickly (Figure 2, lane 2), while the plasmid inside AF-lipoplexes containing an amount of AF below 4.5 μg was protected completely

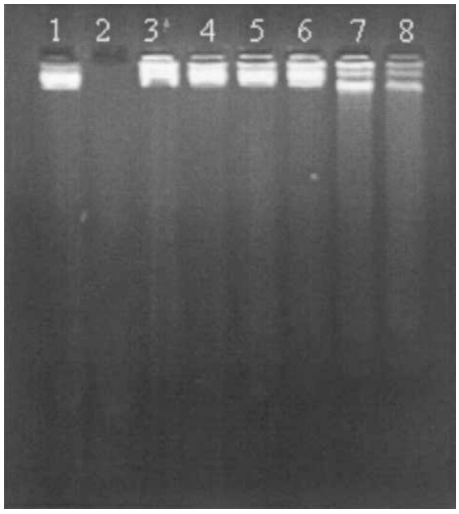

Figure 2 *Protection assay. Stability to degradation by DNase I of free DNA or DNA formulated in AF-lipoplexes with different amounts of the ligand. Untreated DNA (lane 1), DNA treated with DNase: naked plasmid (lane 2), DNA inside lipoplexes prepared with zero (lane 3), 0.1 (lane 4), 1 (lane 5), 4.5 (lane 6), 9 (lane 7) and 18 (lane 8) μg AF/μg DNA.*

(Figure 2, lanes 3–6). Quantification of the intensity of the bands in the gel, also showed some DNase degradation (50% compared to naked DNA) in complexes with 9 μg of AF (Figure 2, lane 7) and practically total degradation (70%) when lipoplexes were prepared with 18 μg of the ligand (Figure 2, lane 8).

Effect of asialofetuin on lipofection activity in HepG2 cells

Figure 3 shows the luciferase gene expression of complexes formulated at 4/1 (+/−) charge ratio and 1 μg of pCMVLuc, with different amounts of AF. Complexes containing the ligand in the range from 0.01 to 9 μg AF/μg DNA gave always higher values of gene expression compared to plain lipoplexes (without ligand), with the optimal amount being 1 μg. This complex showed a 16-fold higher transfection activity than non-targeted lipoplexes. Complexes prepared with amounts of the ligand above 9 μg decreased gene expression dramatically. In fact, levels of luciferase expression obtained with AF-lipoplexes containing 36 μg AF/μg DNA were 4-fold lower than that obtained with plain complexes. No gene expression was detected with the naked plasmid.

Specificity of targeting to the asialofetuin receptor

To elucidate whether the uptake of AF-lipoplexes, prepared at 4/1(+/−) charge ratio and with 1 μg of plasmid DNA, is mediated via interaction with the AF receptor, a competitive inhibition experiment was performed in the presence of an excess of the free ligand. In addition, HeLa cells, which are deficient in ASGPr, were used in this experiment. Figure 4 shows that the treatment of HepG2 cells with 1 mg of free AF previous

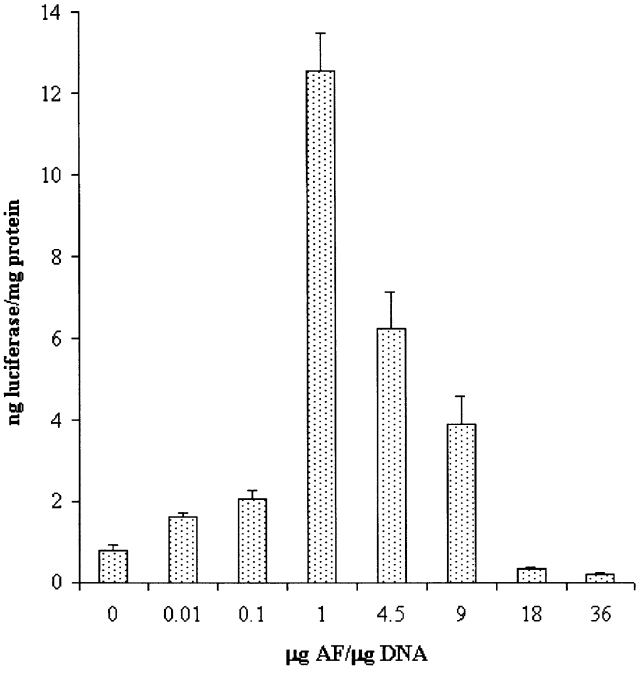

Figure 3 *In vitro transfection activity by AF-lipoplexes. Transfection of HepG2 cells by plain and AF-lipoplexes as a function of the amount of the ligand. The data represent the mean ± s.d. of three wells and are representative of three independent experiments.*

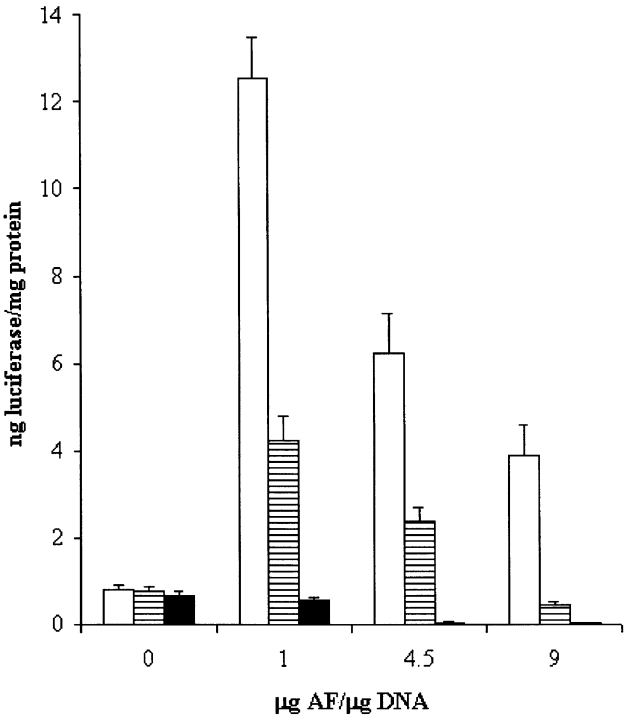

Figure 4 *Competition assay. Gene expression by plain and AF-lipoplexes in HepG2 cells in the absence (□) or presence of an excess of free AF (▤) and in HeLa cells (■). The data represent the mean ± s.d. of three wells and are representative of three independent experiments.*

to the addition of complexes significantly inhibited gene expression of AF-lipoplexes but not that of plain complexes. Lipoplexes containing 1, 4.5 and 9 μg of AF showed a 3-, 3- and 9-fold decrease in the levels of gene expression, respectively. On the other hand, the same lipoplexes presented a 23-, 156- and 194-fold decrease, respectively, when added on HeLa cells, however, no differences were observed with plain complexes. These results support the hypothesis that AF-lipoplexes are recognized by the ASGPr on HepG2 cells.

Effect of protamine sulfate on gene expression mediated by AF-lipoplexes

AF-lipoplexes, with 1 μg of DNA at 4/1 (+/−) charge ratio, in the absence or presence of different amounts of protamine sulfate, were incubated with HepG2 in the presence of 60% FBS. Figure 5 shows the gene expression as a function of the amount of the condensing peptide. Independent of how much protamine was used in the formulation of complexes, the presence of AF resulted always in higher values of gene expression compared to plain lipoplexes. The optimal amount of protamine resulted to be 0.4 μg/μg DNA and gene expression was calculated to be about 5-fold over the value obtained with AF-lipoplexes in the absence of the peptide. This value was 30 times higher than the one obtained by plain lipoplexes with the same amount of protamine. Peptide amounts above 0.4 μg decreased the gene expression dramatically. Table 1 shows the particle size of plain and AF-lipoplexes at 4/1 (+/−) charge ratio, containing 1 μg of the ligand, 1 μg of pCMVLuc and different amounts of protamine sulfate per microgram of DNA. By increasing

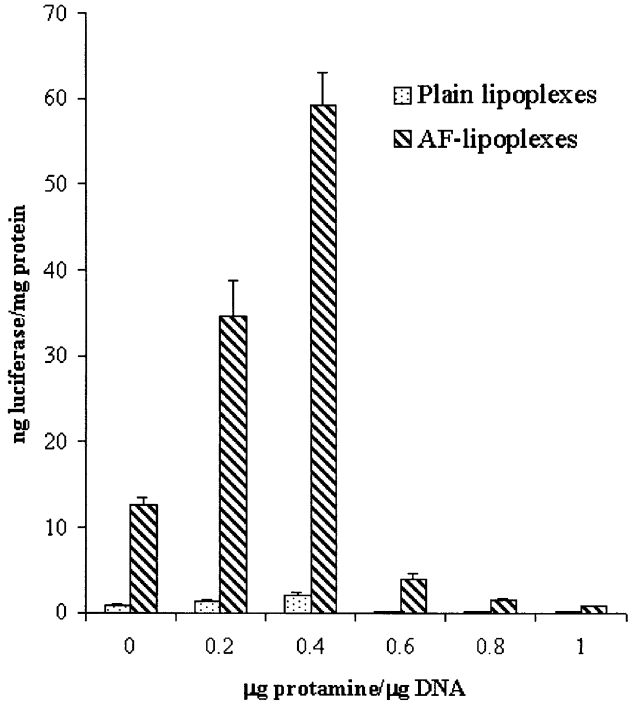

Figure 5 *In vitro gene expression by protamine-AF-lipoplexes. Transfection activity of plain and AF-lipoplexes containing 1 µg AF/µg DNA as a function of the amount of protamine sulfate. The data represent the mean ± s.d. of three wells and are representative of three independent experiments.*

the amount of the peptide to 0.6–0.8 µg the smallest size was obtained. Addition of higher amounts of protamine did not lead to smaller complexes.

Cell viability assay

Cell viability following transfection was assessed to evaluate whether plain and AF-lipoplexes (containing 1 µg AF/µg DNA), formulated with different amounts of protamine, were toxic to the cells. The Alamar blue assay showed a viability higher than 95% in all transfected wells (Figure 6). The relative cytotoxicity of lipoplexes was also assessed by the total amount of extractable cellular proteins in the cell lysate per well, and confirmed the results with the Alamar blue assay.

In vivo studies

In order to verify whether our complexes, prepared as described in 'Materials and methods' could be used for *in vivo* gene delivery, we evaluated the efficacy of protamine-AF-lipoplexes injected systemically into mice. The size and zeta potential of these complexes resulted to be 184 nm and 26 mV, respectively. As shown in Figure 7, these complexes improved significantly luciferase expression, which was calculated to be about 12- and 3-fold higher than plain and AF-lipoplexes, respectively. No transfection activity was detected by injecting the naked DNA. *In vivo* competition experiments were performed by previous pretreatment of mice with free asialofetuin (25 mg/kg), leading to a clear inhibition of the gene expression in the liver by AF-lipoplexes. This fact corroborates that the *in vivo* uptake is also mediated by the ASGPr.

The efficiency of transfection mediated by protamine-AF-lipoplexes was also evaluated by monitoring the expression of green fluorescent protein (GFP) intensity and comparing this efficiency with that of transfection mediated by plain lipoplexes and naked DNA. For this purpose, frozen liver sections from mice injected with PBS (controls), DNA alone, plain and protamine-AF-lipoplexes were examined under a fluorescence microscope. The reporter plasmid pCMV-GFP gave similar results to the luciferase activity, showing that fluorescence by protamine-AF-lipoplexes is superior to that of plain lipoplexes. No fluorescent cells were observed in control animals or in mice administered with GFP plasmid alone (Figure 8). In the same figure is also shown the absence of liver damage, given that no obvious histological changes were seen in liver sections of animals injected with plain and protamine-AF-complexes compared to control mice. The hepatocytes were identified by their characteristic morphology.

Discussion

The existence of ASGPr in hepatocytes facilitates the feasibility of lipoplexes-directed therapy to the liver. The rate and extent of liposomal uptake by this organ is highly variable and dependent on the size, charge, composition, rigidity and other physicochemical properties of the particles. It is already known that delivery to parenchymal cells *in vivo* is favored by small liposomes.[6] For most vectors, high efficiency of transfection correlates with a large excess of cationic charges,[38–40] however, this large excess *in vivo* facilitates non-specific interactions with many undesired elements such as extracellular matrix and negatively charged serum components. Taking this into account, the first aim of this work was to optimize the size and the zeta potential of AF-lipoplexes for maximum uptake by the liver after systemic administration.

As shown in Figure 1, AF amounts below 1 µg lead to AF-lipoplexes of approximately 300 nm with a positive value of the zeta potential, showing that addition of the

Table 1 Size of protamine-AF-lipoplexes. Particle size of plain and AF-lipoplexes as a function of the amount of protamine. Results are expressed in nm as the mean ± S.D. of three experiments

	Protamine sulfate (µg)											
	0		*0.2*		*0.4*		*0.6*		*0.8*		*1*	
	Mean	*s.d.*	*Mean*	*s.d.*	*Mean*	*s.d.*	*Mean*	*s.d.*	*Mean*	*s.d.*	*Mean*	*s.d.*
Plain lipoplexes	242	6	206	7	155	5	120	22	125	45	136	81
AF-lipoplexes	302	5	252	7	181	9	165	4	149	3	155	3

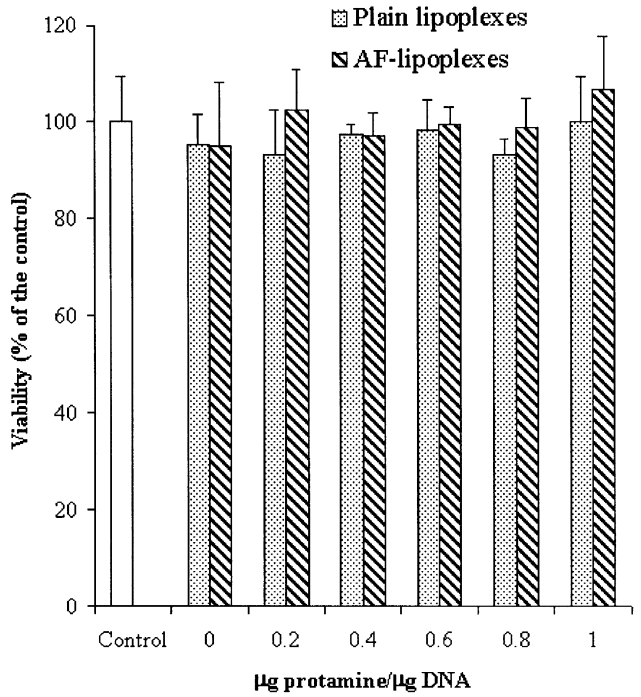

Figure 6 *Cell viability. Effect of plain and AF-lipoplexes on the viability of HepG2 cells. The data represent the mean ± s.d. of three wells and are representative of three independent experiments.*

ligand increases only slightly the particle size of plain complexes and at the same time results in a decrease of the surface charge. This effect is most likely due to the negatively charged amino acids of the protein interacting with the cationic liposomes. As it was expected, amounts of the ligand between 4.5 and 9 µg, leading to a zeta potential close to the electroneutrality, produced aggregates. Amounts of AF above 18 µg lead to complexes with a negative surface charge and particle size even smaller than plain complexes, possibly due to a lack of interaction of DNA with the cationic lipid as a consequence of the presence of a high amount of AF.

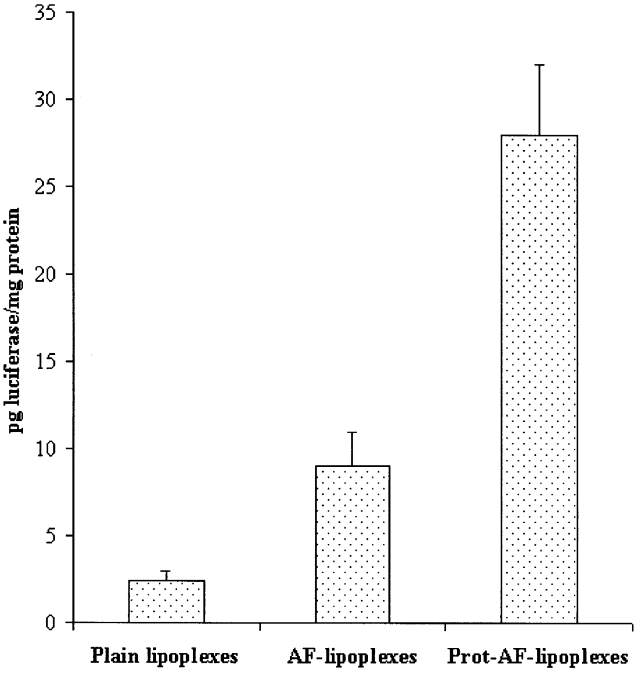

Figure 7 *In vivo transfection activity. In vivo transfection activity of plain, AF- and protamine-AF-lipoplexes. Complexes in the presence of the polycation were prepared with 0.4 µg of protamine as described in Materials and methods.*

In order to avoid *in vivo* degradation by DNases, we wanted to know if the plasmid is protected inside our AF-lipoplexes. When the DNA itself was treated with DNase I, under physiological conditions, complete degradation occurred (see Figure 2, lane 2). Complete protection of DNA inside the complexes was observed when amounts of AF between 0.01 and 4.5 µg were used (Figure 2, lanes 3–6). In these complexes, the interaction of DNA with the lipid is strong enough to achieve protection. When the amount of the ligand was 9 µg or higher, some degradation of the plasmid occurred as a result of the competition of AF with DNA for interaction with the lipid (Figure 2, lanes 7 and 8). In this case, there is more free DNA, which is not complexed, and therefore exposed to DNase.

The association of AF with DOTAP/Chol/DNA complexes increased luciferase expression compared to plain lipoplexes when amounts of AF between 0.01 and 9 µg were used (Figure 3). The greatest enhancement was obtained by complexes containing 1 µg of AF (16-fold). These lipoplexes have a relatively small particle size, which is in accordance with the results obtained by Machy *et al*,[41] who observed that small particles may be more readily endocytosed by the cells, resulting in increased levels of transgene expression. Another possible explanation for this enhancement is that non-specific interactions of plain lipoplexes with negatively charged macromolecules in serum most likely prevent their binding to the cells, while AF-lipoplexes can interact with cell surface receptors. DOTAP/Chol liposomes bind AF through the negatively charged groups present in the ligand at physiological pH, and the resulting structure forms a complex with DNA through a charge–charge interaction. Association of AF also appears to facilitate the internalization of the complex, due to the ability of the ligand to stimulate endocytosis.[42] It is also worth noting that, although other authors have reported gene expression in HepG2 cells by AF-lipoplexes,[29] the enhancement achieved over plain complexes was much lower (1.5-fold increase) than the one obtained with our formulations. Moreover, all our *in vitro* experiments were performed in the presence of 60% FBS, as in our previous work.[43,44] When the amount of AF was above 9 µg, transfection activity by AF-lipoplexes was lower than plain complexes, in spite of the presence of the ligand.

The competitive inhibition of transfection observed by AF-lipoplexes in HepG2 cells in the presence of an excess of free AF (Figure 4) supports the hypothesis that these complexes are recognized by AF receptors on the cells surface, which in turn facilitate receptor-mediated endocytosis. Transfection was not inhibited completely, despite the 1000-fold excess of free AF. This may be the result of the higher avidity of the interaction of multiple AF molecules on lipoplexes with their receptors. It may also be caused in part by the charge interactions between positive lipoplexes and the negative surface charge of cells. Gene delivery and expression by plain lipoplexes

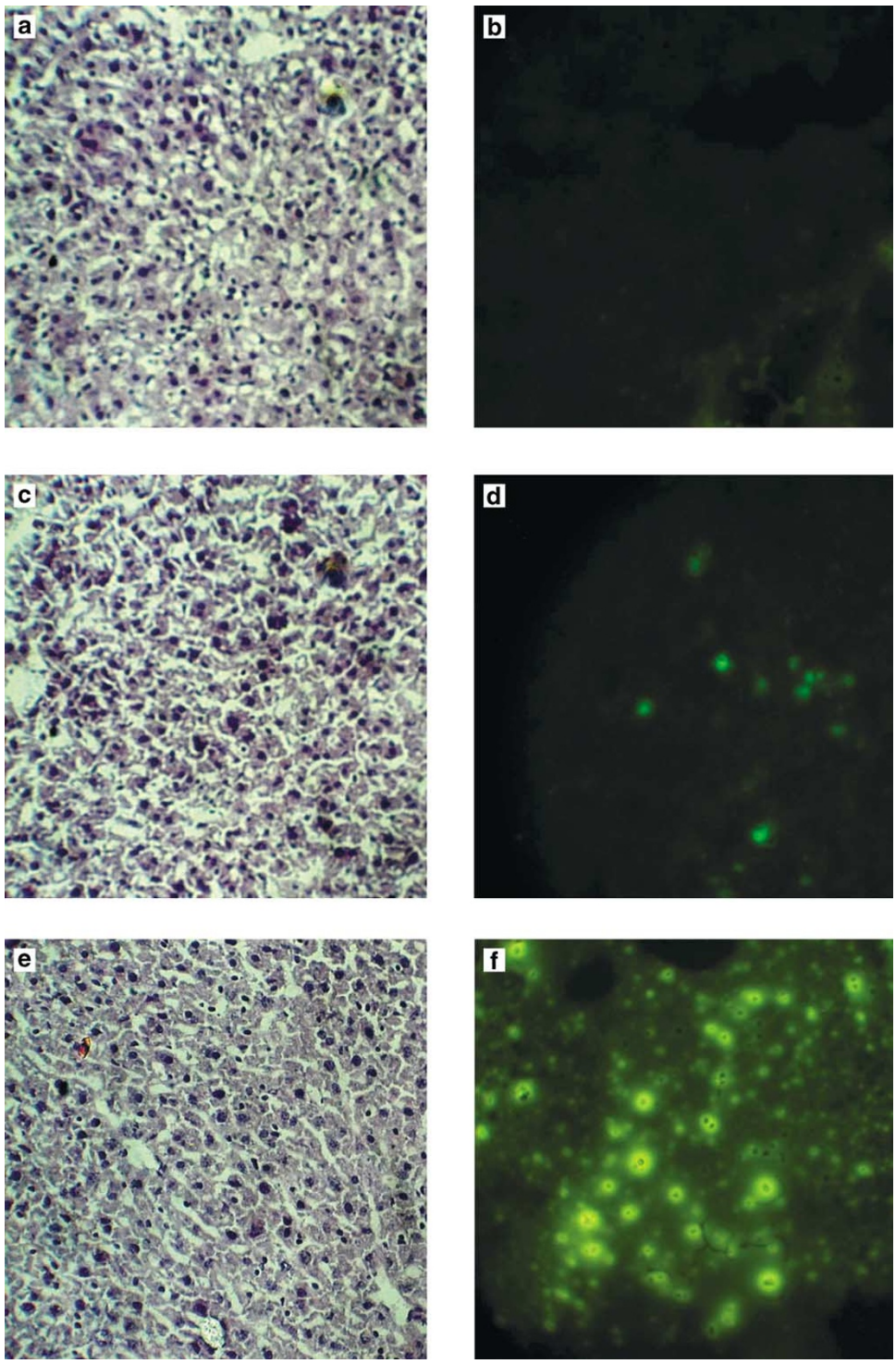

Figure 8 *Histological analysis. Histologic evaluation of GFP gene expression in hepatocytes after tail vein injection of PBS and naked DNA (a,b), plain (c,d) and protamine-AF-lipoplexes (e,f). Cells under phase contrast microscopy (a,c,e) and under fluorescence microscopy (b,d,f). A representative overview is shown.*

did not show changes in the presence of such excess of the ligand. On the other hand, transfection activity of AF-complexes in HeLa cells (defective in ASGPr) also decreased, compared to that obtained in HepG2 cells, remaining in all cases lower than that of plain lipoplexes, which can be bound to the cells by non-specific interactions. On the other hand, it should be noted that an increased charge ratio in the complexes does not change the inhibitory effect of an excess of AF, not altering the ASGPr-mediated uptake. These observations lend support to the role of AF receptors in the mechanism of transfection by AF-lipoplexes. *In vivo* competition experiments also corroborated this mechanism.

The next step we considered in this study was to precondense the DNA with protamine sulfate before the addition of the ligand, in order to have smaller complexes for maximum uptake by the liver after intravenous administration. Figure 5 shows a dose-dependent gene expression by protamine induced in plain and AF-lipoplexes. Maximum level of transfection was obtained at protamine concentration of 0.4 μg per microgram of DNA. Under these conditions, the diameter of the AF-lipoplexes was reduced from 302 to 181 nm (see Table 1). Protamine maintains its ability to condense the DNA without reducing further interactions with the cationic lipids, acting synergistically with it to enhace gene transfer. The use of this polycation could improve the *in vivo* activity of lipoplexes by protecting the DNA from the degradation by nucleases. Also, the presence of nuclear localization signals in its amino acid sequence can potentiate gene expression by increasing the nuclear translocation of DNA.[34] Continuous increase in the amount of protamine was not associated with a further increase in the levels of expression. On the contrary, gene expression was even lower than AF-complexes without the peptide. Probably, the neutralizing effect of an excess of protamine on DNA lead to charge-neutralization, avoiding further interactions with the cationic lipid and the ligand. The polycation itself in combination with DNA had no significant effect on gene expression. The differences in transfection activity between these sets of complexes cannot be explained by different toxicities of the formulations, since cell viability was similar in all cases (Figure 6).

Although a number of gene delivery systems targeted to the ASGPr have been designed, their *in vivo* efficiency has been poorly documented. As shown in Figure 7, protamine-AF-lipoplexes increase gene expression in the liver compared to plain and AF-lipoplexes. The AF molecule, in addition to acting as a ligand, neutralizes part of the positive charge on the liposomes, possibly leading to diminished interactions of the AF-lipoplexes with polyanionic molecules in serum. The development of a different structure in these AF-lipoplexes, together with the assistance afforded by protamine in the nuclear transport of plasmid and the reduction of the size, are likely to be additional reasons why the transfection activity is enhanced. AF-lipoplexes acquired resistance to nuclease attack *in vitro* (see Figure 2), which might be correlated with *in vivo* protection of DNA against endogenous DNases. Moreover, in these lipoplexes the addition of protamine has helped to reduce the need for a large amount of cationic lipid. Consequently, no signs of toxicity were evident when protamine-AF-lipoplexes were injected into mice compared to plain complexes. Results shown in Figure 8 corroborated this observation. Intense green staining was evenly distributed throughout the liver, when mice were injected with protamine-AF-lipoplexes compared to plain complexes. The extent of staining is consistent with the luciferase levels, positively staining cells being mainly hepatocytes. None of the liver sections observed showed evidence of necrosis.

In summary, the novel protamine-AF-lipoplexes developed in this study, have improved transfection activity both *in vitro* and *in vivo*. Although various methods have been used for the preparation of polycation–ligand–DNA complexes, covalent binding is usually needed, and insoluble aggregates are formed unless the concentration of the polycation or DNA is minimized. The complexes in this study, however, were prepared by simple mixing of the peptide and the ligand, without having problems of aggregation. Although previous studies have shown that the association of AF with lipoplexes enhances transfection, this is the first demonstration of the efficacy of the combination of the condensing action of protamine and the targeting effect of the ligand *in vivo*. The synthetic nature of this system provides the potential of flexibility in terms of composition, and the capability of inexpensive and large-scale production of the complexes. Further improvement of this non-viral vector, such as prolonged expression of the transgene, could bring us into the range of a gene delivery system with clinical benefit.

Materials and methods

Materials

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). AF Type I, *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES) and protamine sulfate (derived from salmon) were obtained from Sigma (Madrid, Spain). Alamar Blue dye was purchased from Accumed International Companies (Westlake, OH, USA), and DNase I and ethidium bromide from GibcoBRL Life Technologies (Barcelona, Spain). The plasmids, pCMVLuc (VR-1216) and pCMV-GFP (Clontech, Palo Alto, CA, USA), encoding luciferase and GFP, respectively, were used for carrying out the transfection experiments. Plasmids were amplified in *E. coli*, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Purity was confirmed by 0.8% agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm. The reagents used in the histological analysis of GFP gene expression were from Merck (Barcelona, Spain).

Cell culture

HepG2 (human hepatoblastoma) and HeLa (human cervix carcinoma) cells were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium-high glucose (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM) (all from GibcoBRL Life Technologies, Barcelona, Spain).

Preparation of lipoplexes

Firstly, DOTAP/Chol (1:0.9 molar ratio) liposomes were prepared by drying a chloroform solution of the lipids by rotary evaporation under reduced pressure. Then, the film was hydrated with a 10 mM HEPES, 10% (w/v) glucose buffer (pH 7.4), to give a final concentration of 10 mM DOTAP/9 mM Chol. The resulting multilamellar vesicles were extruded 5 times through polycarbonate membranes with 100 nm pore diameter using a Liposofast device (Avestin, Toronto, Canada), to obtain a uniform size distribution and filter-sterilized (Millex 0.22 μm, Millipore, Bedford, MA, USA). Liposomes were

stored at 4°C under nitrogen and were used within 1 month after preparation.

Complexes for *in vitro* experiments were prepared at a 4/1 (+/−) charge ratio by sequentially mixing 100 μl of a solution of 10 mM HEPES, 10% (w/v) glucose buffer (pH 7.4) without (plain lipoplexes) or with a variable amount of AF (AF-lipoplexes) and 22.8 nmol of the DOTAP/Chol liposome suspension. After incubating for 15 min at room temperature, 100 μl of water containing 1 μg of pCMVLuc were added and gently mixed. Protamine-AF-lipoplexes were prepared by pre-complexation of plasmid DNA with different amounts of protamine, followed by the addition of cationic liposome-AF complexes. Lipoplexes for *in vivo* experiments were prepared at a 5/1 (+/−) charge ratio and contained 19 mM of total lipid (DOTAP/Chol). Protamine-AF-lipoplexes were formulated with 0.5 μg of AF and 0.4 μg of protamine per microgram of DNA. The final concentration of plasmid in the complexes was 0.3 mg/ml. The lipid to DNA charge ratio was calculated as the mole ratio of DOTAP (one charge per molecule) to nucleotide residue (average MW 330). The protocol used for the preparation of these complexes implied also the pre-complexation of plasmid DNA with protamine followed by the addition of preformed cationic liposomes-AF-complexes, prepared as described above. The lipoplexes resulted to be of easy preparation and very stable, without having problems of aggregation, which was one of the main reasons to choose this formulation for *in vivo* experiments.

Cell culture transfections

For transfection, 10^5 cells were seeded in 1 ml of medium in 48-well culture plates (10 mm well diameter; Iwaki, Japan) 24 h before addition of the complexes and used at approximately 80% confluency. Cells were washed twice with DMEM without antibiotics and then 0.3 ml of fetal bovine serum and 0.2 ml of complexes were added gently to each well. After a 4-h incubation in 60% FBS (at 37°C in 5% CO_2) the medium was replaced, and the cells were further incubated for 48 h in medium containing 10% FBS.

In vitro transfection activity

After 48 h, cells were washed with phosphate buffered saline (PBS) and lysed using 100 μl of reporter lysis buffer (Promega) at room temperature for 10 min, followed by two alternating freeze–thaw cycles. The cell lysate was centrifuged for 2 min at 12 000 *g* to pellet debris. Then, 20 μl of the supernatant was assayed for total luciferase activity using the luciferase assay reagent (Promega), according to the manufacturer's protocol. A luminometer (Sirius-2, Berthold Detection Systems, Innogenetics, Diagnóstica y Terapéutica, S.A., Barcelona, Spain) was used to measure luciferase activity. The protein content of the lysates was measured by the DC Protein Assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The data were expressed as ng or pg of luciferase (based on a standard curve for luciferase activity) per milligram of protein.

DNase I protection assays

To evaluate the role of our complexes in the protection of DNA, the naked plasmid and lipoplexes with different amounts of AF were incubated in the presence of the enzyme. DNase I (1 unit per μg of DNA) was added to 2.5 μg (DNA) of each sample and the mixtures were incubated at 37°C for 30 min. Two microliters of EDTA (0.5 M) were immediately added to stop DNase degradation. Then, sodium dodecyl sulfate (SDS) was included to a final concentration of 1% to release DNA from the complexes. Finally, samples were analyzed by 0.8% agarose gel electrophoresis and the integrity of the plasmid in each formulation was compared with untreated DNA as a control.

Cell viability assay

Cell viability was quantified by a modified Alamar Blue Assay.[45] Briefly, 1 ml of 10% (v/v) Alamar Blue dye in DME-HG supplemented with 10% (v/v) FBS medium was added to each well 48 h after transfection. After 2.5 h of incubation at 37°C, 200 μl of the supernatant was assayed by measuring the absorbance at 570 and 600 nm. Cell viability (as a percentage of control cells) was calculated according to the formula $(A_{570}–A_{600})$ of treated cells $\times 100/(A_{570}–A_{600})$ of control cells.

In vivo gene expression

Female Balb-c (8–10 weeks of age) mice were purchased from Harlan Ibérica Laboratories (Barcelona, Spain). All animals were studied in accordance with guidelines established by Directive 86/609/EEC and with the approval of the Committee on Animal Research at the University of Navarra, Pamplona (033/00). Individual mice in groups of eight were injected via the tail vein with 60 μg of DNA formulated in plain, AF- or protamine-AF-lipoplexes in a total volume of 200 μl 5% w/v glucose. Twelve hours following iv injection, the mice were killed with a sodium pentobarbital overdose. The liver was collected and washed with cold saline twice. The organs were homogenized with lysis buffer (Promega) using a homogeneizer (Mini-Beadbeater™ , BioSpec Products, Inc., Bartlesville, OK, USA) and centrifuged at 12 000 × *g* for 3 min at 4°C. Finally, 20 μl of the supernatant was analyzed for luciferase activity as described previously. In the histological analysis, the livers were dissected and immediately frozen. Six-micrometer cryosections were made using a cryostat (Microm, HM 505 E) and regular hematoxylin–eosin staining was used. The sections were examined in a microscope (Olympus CH-40, Japan) with fluorescence source (Olympus U-RFLT50, Japan), equipped with a digital camera (Olympus Camedia C-3030 zoom), at an original magnification of × 200.

Particle size and zeta potential measurements

The particle size of complexes was measured by dynamic light scattering, and the overall charge by zeta potential measurements, using a particle analyzer (Zetamaster, Malvern Instruments, Spain). Samples of the prepared complexes (2.5 ml) were measured 3 times for 60 s at 1000 Hz and an electric current of 3 mA with zero field correction.

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