Cationic liposomes as DNA delivery system. Characterization of SAINT-mediated gene transfer
Audouy, Sandrine Anne Louise

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Chapter 7

Summary

The work presented in this thesis was aimed at characterizing cationic liposome-mediated transfection and, more specifically, investigated the properties of the synthetic cationic lipid SAINT-2. In this final chapter, the data obtained with SAINT-2 containing liposomes will be summarized and discussed. Some perspectives concerning the future application of cationic liposomes in different gene therapy approaches will also be presented.

Over the last 10-15 years, considerable progress was made in the development of competent DNA delivery systems. This has paralleled the course of preclinical and clinical studies employing gene therapy, setting increasingly higher requirements in terms of safety and efficiency of the vectors. In particular, the development of cationic liposomes for such a purpose has generated extensive work and these systems may well play an indispensable role in gene therapy. The structure and physico-chemical properties of these complexes with DNA (lipoplexes) have been elucidated, as well as the (intra-)cellular events leading to transgene expression in vitro. The potential of lipoplexes for therapeutic purposes has been tested in diverse animal models and is currently under clinical investigations. As an introduction, Chapter 1 situates cationic liposomes in the general context of gene therapy and presents progresses in research on lipoplexes in vivo. In chapter 2, various parameters, such as lipid:DNA ratio, size of the lipoplexes or the effect of serum, influencing lipofection in vitro and in vivo, are reviewed. Recent improvements in lipoplex technology regarding efficiency, stabilization and targeting are also described.

The main objective of the work presented in this thesis was to improve our understanding of the parameters that modulate SAINT-mediated transfection. Also, the potential of SAINT-2 cationic liposomes as gene carriers for gene therapy applications was tested. In chapters 3 and 4, we studied several aspects of transfection in vitro, employing different cell lines. The transfection efficiency of numerous cationic lipid-based formulations is impaired by the presence of serum. Therefore, the influence of this factor on SAINT lipoplexes was investigated (chapter 3). It was established that transgene expression levels obtained in several cell lines with SAINT lipoplexes, in contrast to Lipofectin, were not heavily affected by the presence of serum. The behavior in serum was not only dependent on the nature of the cationic lipid but also on the helper lipid included in the liposomes, and interestingly on the cell line tested. Transfection-related cytotoxicity was reduced when serum was present. At the lipoplex level, we observed a direct stabilizing effect of serum, preventing aggregation of the particles and preserving transfection activity of the particles over time. This process at different serum concentrations is depicted in Figure 4.3. As an example, the titration of lipoplexes in serum was carried out by mixing lipoplexes with different concentrations of serum. Significant differences in efficiency were observed, with higher transfection efficiency obtained at lower serum concentrations. In order to improve the overall transfection efficiency, particularly in situations of higher serum content, a two-step transfection approach was developed. First, the lipoplexes were incubated with serum to stabilize them, then cells were exposed to the lipoplexes in serum-containing medium. Using this method, transfection efficiency was significantly enhanced compared to the standard procedure. The resulting PEGylated lipoplexes, allowing efficient cell penetration, were found to transfect cells at much lower concentrations of lipoplexes and serum. This approach opens new avenues for the use of lipoplexes in gene therapy applications, particularly in situations where serum is present. The study of the interaction of lipoplexes with serum is a key aspect in the development of effective gene delivery systems.
particles over time. Altogether, the data showed that serum affected the transfection process at different stages during lipoplex-cell interaction. The insensitivity of SAINT transfection toward the presence of serum is an interesting finding, but extrapolation to an in vivo situation should be considered with much care. The stability of lipoplexes in mouse serum has been related to in vivo transfection efficiency. SAINT lipoplexes were stabilized in 10% serum but this does not exclude destabilization at higher serum concentrations.

In order to develop a 'stealth' SAINT formulation, a small percentage of poly(ethylene glycol)-modified lipids was introduced in SAINT lipoplexes, and the effect of this modification was examined in an in vitro system (chapter 4). The PEG technology was initially developed to prolong the circulation time of classical liposomes after i.v. administration and is based on the hydrophilicity of the PEG groups, masking the particle. It was speculated that, similarly, a PEG-coating could stabilize lipoplexes and at the same time reduce its non-specific interactions with cells/ blood components. This hypothesis was tested with four species of PEGylated lipids that differed by the length of their acyl chain. This variation modulates anchoring properties in a lipid bilayer, short-chain PEG-lipids exchanging more rapidly than long-chain ones in the presence of an acceptor membrane. Addition of PEG-lipids did not compromise lipoplex formation but reduced the average size of the lipoplexes. An important finding was that transfection properties of SAINT liposomes in B16-F10 cells were dramatically affected by the introduction of PEG-lipids. When the PEG-lipids were included in the liposomes, transfection with the resulting PEG-lipoplexes was totally inhibited for the long chain PEG-lipids and to a large extent for the short-chain ones. By adding the PEG-lipids concomitantly to lipoplex formation, we observed no inhibition of transfection with the long chain PEG-species whereas the inhibition obtained with the short-chain species was not significantly different. PEG-lipoplexes were quickly taken up in the cells, using either method of lipoplex PEGylation, suggesting a different intracellular pathway for PEGylated vs. non-PEGylated lipoplex, i.e. one leading to degradation and the other allowing escape of the DNA before degradation. The net positive charge of the PEG-lipoplexes was identified as the parameter determining cell-lipoplex interaction, as the binding of PEG-lipoplexes could be reduced by lowering the (+/-) ratio from 4:1 to 2:1. Taken together, these data revealed a profound influence of PEG-lipids on the lipoplexes and on the transfection process. Interestingly, the cellular binding of the PEG-lipoplexes was modulated by the net charge ratio of the lipoplexes and could only be inhibited when decreasing this ratio.

The studies presented in chapters 5 and 6 investigated the ability of SAINT lipoplexes to transfect a tumor in vivo. To this end, a murine melanoma model presenting a
subcutaneous tumor was used, and the lipoplexes were applied by local and systemic administration. Following intravenous injection (chapter 5), firstly safety of the SAINT lipoplex treatment was evaluated. At the concentrations required for in vivo injection, lipoplexes prepared in 0.9 % NaCl rapidly precipitated in vitro and were found to induce mild toxicity in healthy BALB/c mice in vivo. Apathy, fur ruffling, leukopenia (drop in the number of circulating white blood cells: WBC) and hypertrophy of the spleen were evidenced at a dose of 1 µg DNA (combined with 15 nmol SAINT liposomes), but these symptoms resolved within a few days. Preparing the lipoplexes in 5% glucose resulted in an improved stability (no precipitation in vitro) and drastically decreased the toxicity of SAINT lipoplexes. Mice received lipoplexes containing 5 µg and 25 µg DNA without notable changes in the WBC counts and without apparent liver pathology. Still, the red pulp in the spleen of all animals was slightly increased, and mice injected with 25 µg DNA displayed transient fur ruffling. We then studied the biodistribution of SAINT lipoplexes in mice bearing a subcutaneous tumor, and how this was influenced by PEGylation of the lipoplexes. One important question was whether the PEG-lipids could prolong the circulation time of SAINT lipoplexes and thereby enhance their uptake into the tumor or not. Following radiolabeled lipoplexes after i.v. injection, a rapid blood disappearance of SAINT lipoplexes was observed, illustrating a general feature of this class of delivery agents. After 10 min, no radioactivity was detected in the blood for non-PEGylated lipoplexes or lipoplexes containing 5% PEG-lipid. However, the radioactivity still present in the blood after the same time was slightly increased for lipoplexes containing 10% PEG-PE compared to non-PEGylated lipoplexes. Organ distribution studies revealed that SAINT lipoplexes, with or without PEG-lipids, were taken up in the liver and spleen within 10 minutes, contrasting with many formulations which usually display an intermediary step of lung accumulation. This finding may be explained by a rapid destabilization of the lipoplexes, which could depend on (i) the lipoplex formulation, including DOPE as helper lipid, and (ii) a relatively low net positive charge (with a +/− ratio of 2:1). The amount of radioactivity detected in the tumor was negligible and optimization apparently is needed to adapt SAINT lipoplexes to tumor delivery by systemic administration. This should include approaches to prolong the circulation time of the particles by reducing non-specific interactions and uptake by macrophages. PEG-lipids bearing a longer hydrophilic chain (PEG5000) than the ones used in our experiments may be useful for this purpose. Indeed, our data (chapters 4 & 5) suggest that the electrostatic interactions between lipoplexes and cells (in combination with blood components) are difficult to block by incorporation of PEG5000-lipids. The failure to detect expression of the transgene in lung, liver and spleen could indicate that the detection method used was not sensitive enough, or that the localization of the lipoplexes suggests that redistribution into macrophages. In order to obtain the behavior of our system more accurately, their biodistribution was tested in a murine subcutaneous intratumoral model. The lipoplex is still present at the injection point. Quantitative transfection analysis to further enhance expression was investigated. The L929 model. The lipoplex was injected after intratumoral injection and successfully demonstrates a therapeutic potential.

Perspective

The results demonstrate the attractiveness of several approaches. The possibility of lipoplexes to target based systems, and the potential of several applications need to be explored.
Summary and perspectives

The results obtained with SAINT lipoplexes that are presented in this thesis demonstrate the potential of the SAINT-2 amphiphile in therapeutic gene delivery approaches. However, our data also reflect limitations associated with cationic lipid-based systems and raise several points about their future development and applications in gene therapy. Among the vectors available, non-viral systems remain attractive compared to viral ones, notably because of ease and low cost of production,
the convenience of use, the relative safety of administration with the possibility to repeat injection.
For the time being, realistic indications for cationic lipid-based devices are limited to local administration. Preclinical studies have validated the use of cationic liposome/DNA complexes for treatment of cystic fibrosis and certain tumors, but such successes have been translated in the clinic only to a limited extent. The demonstration of transgene expression in patients, following pulmonary or tumoral administration of lipoplexes, is an encouraging start. The proteins expressed appeared functionally active, which indicates that the lack of therapeutic benefit could be due to the insufficient level of expression. Several phase II trials are still ongoing and their outcome will provide complementary information to evaluate cationic liposomes for clinical application. Other gene therapy applications that could make use of cationic liposomes are vaccination, treatment of hemophilia B and cardiovascular diseases. In these cases, local transgene expression is required in the skin, muscle or vascular wall and these tissues are efficiently transfected by lipoplexes as demonstrated by in vivo studies. Intravenous injection represents a further step, and may be proposed for lung diseases, due to the intrinsic lung targeting property of lipoplexes.
Improvement of efficiency and targeting of transfection remain major challenges in the field of non-viral vectors. These are ‘sine qua non’ conditions for successful application in systemic gene therapy. Next to vector engineering, modifications at the DNA level may prove valuable to reach these goals. For instance, strong non-viral promoters, less susceptible to inactivation by inflammatory cytokines, may prolong the duration of expression and the use of tissue specific promoters is an interesting approach for targeting purposes. Unmethylated sequences on bacterial DNA induce inflammatory responses after lipoplex administration, leading to transgene inactivation. Eukaryotic DNA or DNA methylation could limit the activation of the innate immune system and thereby enhance duration of transgene expression. Parameters such as lipid:DNA ratio and helper lipid, that affect the physical properties of the lipoplexes and their in vivo distribution and efficiency, should be carefully optimized for each cationic lipid and therapeutic indication. The vector should combine condensing, protecting, stabilizing and targeting properties, with retention of transfection activity. It is thus not excluded that cationic liposomes realize fully their potential in combination with polycations or peptides confering additive properties to the system. Polycations can improve stability and efficiency of the lipoplexes and a targeting moiety can be easily attached to their structure. Custom-made peptides including DNA binding, tissue targeting and nuclear localization signals also represent good candidates to be included in the development
Summary and perspectives

The possibility to use liposomes is limited to the targeting of cationic liposomes in tumors, but the extent. The physical property of tumoral liposomes as micelles appeared could be due to ongoing and intrinsic liposomes. The use of cardiovascular, muscle or tumoral lipoplexes as a first step, and the notion of targeting actually includes several aspects. In the case of local administration, targeting may also increase cellular uptake and thus transfection efficiency. After systemic delivery, the aim of targeting can be to selectively increase the accumulation in a specific organ. Research in the field of drug delivery has identified many potential targets and this knowledge could prove highly valuable for gene transfer. As an example, the RGD motif recognizing integrins expressed on the tumor vasculature may be coupled to lipoplexes to target tumors. Targeting may be used to allow crossing of endothelial barriers. For instance, antibody targeting to the transferrin receptor allows transcytosis across the blood brain barrier of liposomes and proteins. Antibody targeting of a lung specific caveolae component can target proteins to the lung through the lung endothelium. We can predict that more organ-specific, endocytotic or transcytotic targets will be identified in the coming years, allowing specific addressing of drug and possibly lipoplexes and passage across endothelial and epithelial barriers.

Finally, it should be kept in mind that a universal vector to serve multiple purposes may not be a realistic option and that each vector may be the answer to a particular indication.