Cationic liposomes as DNA delivery system. Characterization of SAINT-mediated gene transfer

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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. 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 (PEG_{5000}) 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 PEG_{2000}-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 lipoplex was destabilized leading to DNA degradation. The localization of the lipoplexes in liver and spleen without expression of the transgene suggests that the absence of expression in these organs was due to uptake by macrophages. Accumulation in the liver could be turned as an advantage to apply treatment to this organ, but the lipoplexes should then be targeted to hepatocytes in order to obtain transgene expression. The nature of the helper lipid is a key factor for the behavior of systemically applied lipoplexes and replacing DOPE by cholesterol in our system may enhance the circulation time of SAINT lipoplexes, thus modifying their biodistribution and possibly leading to a better transgene expression.

Local administration limits the number of barriers to overcome before reaching the cells of interest. The possibility to use SAINT lipoplexes for local treatment was tested in a mouse melanoma model. The results, presented in **chapter 6**, unambiguously demonstrate the expression of two different reporter genes following intratumoral injection of 5 µg DNA complexed with SAINT liposomes at a ratio of 15:1 nmol/µg. The use of the GFP gene in combination with fluorescently labeled liposomes revealed that diffusion through the tumor of both components had occurred. Whether this observation relies on the tumor model itself or on the SAINT lipoplex is still unclear. Comparable studies in other tumor models should clarify this point. Quantification of expression levels by the luciferase assay showed an increased transfection ability of SAINT lipoplexes compared to naked DNA. In an attempt to further enhance the efficiency of SAINT lipoplexes, the effect of ultrasound was investigated. No influence on the level of transgene expression was found in our model. The level of expression may be enhanced by modulation of parameters such as lipid:DNA ratio or DNA dose. Nevertheless, the demonstration of protein expression after intratumoral injection of SAINT lipoplexes suggests that a therapy may be successfully applied in this model by administration of SAINT lipoplexes containing a therapeutic gene.

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

of efficient and specific vectors. Targeting of the lipoplexes may also involve attachment of antibodies (or fragments) and ligands to the vector, a technology previously developed for conventional liposomes. 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.