Biochemical Pharmacology, Vol. 54, pp. 9–13, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00872-6

COMMENTARY

Long-Term Expression of the Human α1-Antitrypsin Gene in Mice Employing Anionic and Cationic Liposome Vector

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ABSTRACT. The complete process of gene therapy involves three important steps: targeting, delivery, and gene expression. Since each step can be related to the pharmacological concept of affinity, bioavailability, and intrinsic capacity, this commentary examines, from this perspective, the efficiency of anionic and cationic liposomes as vectors for the *in vivo* gene transfer of the human α1-antitrypsin gene. Small liposomes represent the first generation of liposomes destined for the liver parenchymal cell. Although the final efficiency of gene transfer is low, we found that small liposomes are a kind of high-affinity hepatocyte-destined vector because the dose range for mediating the response is three orders of magnitude lower than that used by other procedures. Encapsulated DNA is more efficient than the cationic liposome–DNA complex for *in vivo* gene transfer. This could be due to gene bioavailability, since encapsulated DNA is protected from enzymatic digestion, whereas DNA externally associated with the liposome can be digested before the complex reaches the target cell. However, when the gene transfer efficiencies of anionic and cationic small liposomes were compared, we observed a similar rate of efficiency and potency, since equivalent plasma levels of human protein were observed after the same i.v. dose of recombinant plasmid encapsulated in anionic or cationic liposomes. On the other hand, the elements selected for constructing the expression cassette greatly influence gene expression and the stability of the gene product, and, therefore, the final efficacy is also limited by the intrinsic capacity of a specific expression cassette to express the gene product. BIOCHEM PHARMACOL **54**;1:9–13, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. α1-antitrypsin; gene delivery; gene therapy; hepatocyte; liposome; liver

Gene therapy is a strategy in which nucleic acid is administered to modify the genetic repertoire of somatic target cells for therapeutic purposes [1], in both inherited and acquired diseases. Liver-destined gene therapy has a promising future, since the liver is an organ in which many metabolic diseases are manifested [2, 3], affecting not only the metabolic function of the liver itself but also the ability of the organ to produce secreted serum proteins [3]. However, several questions remain to be answered before genes can be used as pharmaceutical products in clinical practice, mainly in relation to the type of vector that should help to deliver the nucleic acid into the cells. Although a wide variety of vectors for gene transfer have been used for experimental purposes, they can be classified into viral and non-viral systems. The highest number of therapeutic trials (directed against monogenic hereditary disorders or cancer) employed retrovirus or adenovirus as viral vectors, and DNA–liposome complexes as non-viral vectors. The viral vector uses the ability of the virus to express its genetic information within infected cells, although inflammation and antiviral immune responses are known to pose a risk to recipients and constitute a limitation for repetitive treatment. Non-viral vectors are less efficient for gene transfer, but they offer safety as the main advantage; moreover, they allow genes to be formulated as drugs, pharmacologically studied and directly administered to patients in a dose-dependent manner. The complete process of gene therapy involves three important steps: targeting, delivery, and gene expression. Since each step can be related to the pharmacological concept of affinity, bioavailability, and intrinsic capacity, we comment from this perspective on the efficiency of anionic and cationic liposomes as vectors for the *in vivo* gene transfer of the human α1-antitrypsin gene.

TARGETING/AFFINITY

The targeting efficacy of a vector is directly correlated with its ability to reach the cell to which it has been destined, independent of its ability to mediate an effect or not. Affinity is a measure of how avidly a ligand binds to a target receptor, and is characterized by the equilibrium dissociation constant. The reciprocal value of affinity is known as the affinity constant, which indicates the dose size required to produce 50% of the maximal response. In many cases, the targeting efficacy of a vector is studied using reporter

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genes to evaluate the relative number of transfected cells. The procedure, although very interesting at an experimental level, does not project all of its significance at the clinical level, and the pharmacological concept of affinity (or derived parameters) must contribute to evaluating the *in vivo* targeting efficacy between different vectors bearing the same gene construction and destined for the same organ, tissue, or cell.

It is known that multilamellar and large liposomes are taken up mainly by phagocytic cells, and that continuous endothelium of the vascular bed is a major barrier to liposome release from the circulation to many tissues and organs. However, endothelium corresponding to liver sinusoids is discontinuous and presents pores that allow the crossing of particles with diameters of <100 nm, approximately. When administered *in vivo*, large liposomes accumulate in the liver, but their large size limits access to hepatocytes; consequently, liver accumulation is associated mainly with the phagocytic Kupffer cells within the sinusoidal lumen. In contrast, small liposomes (<100 nm diameter) are able to cross the sinusoidal pores and are taken up efficiently by hepatocytes [4, 5]. In effect, they represent the first generation of liposomes destined to liver parenchymal cells, and they have been used as vectors for human α1-antitrypsin gene transfer to *in vivo* hepatocyes [4, 6]. Although the final efficiency of gene transfer is low [6–8], the efficiency of the vector in the different steps of the complete process must be considered. Thus, we found [8] that the treatment allows repetitive doses, that a maximum effect exists, and that small liposomes are a kind of high-affinity hepatocyte-destined vector because the dose range for mediating the response is three orders of magnitude lower than that used by other procedures [9]. Since the *in vivo* administration of low amounts of gene is desirable in gene therapy to minimize and/or avoid secondary effects, studies focused on investigating the affinity of vectors destined for the liver and/or hepatocytes on the basis of pharmacological dose–response trials will be well received, as they may contribute to improving liver gene therapy strategies. It is true that the *in vivo* affinity should be evaluated by an indirect gene expression procedure, although the responses can be normalized to obtain only objective and quantitative data concerning relative vector affinity (gene–vector potency), in a way similar to the concept of drug potency. Since the lipid composition of liposomes can be modified with the aim of increasing the yield of encapsulation and/or improving their delivering ability, it would be interesting to know how variations in the lipid composition of liposomes (including the net liposome charge) are able to modify their hepatocyte destiny affinity and delivering ability.

DELIVERY/BIOAVAILABILITY

The delivery efficacy of a vector is related to its ability to introduce functioning genes into the cells. To achieve this, the vector must be able to: (i) associate DNA (without damage) to the vector and protect it from enzymatic deg radation during transport to the target cells; and (ii) facilitate gene integrity from destined vector to target cell cytoplasm, and DNA transport to the nucleus. In this sense, and based on the pharmacokinetic concept of bioavailability, we could introduce the terms vector and gene bioavailability to describe the proporation of administered vector or gene reaching the systemic circulation or cell nucleus, respectively.

Liposome transport is based on the self-assembling backbone vesicles formed by lipid bilayers entrapping a fraction of surrounding aqueous medium. Therefore, the yield of DNA encapsulation is a function of DNA concentration and the total entrapped volume, which also depends on the lipid composition and/or liposome structure. Interestingly, liposome-based vectors have a low toxicity and induce a low host immune response, as compared with other non-viral vectors such as polylysin–DNA complex and/or viral vectors; usually, however, the incorporated DNA and the transfection efficiency are low. Two main types of liposomes have been used for gene transfer, based on lipid composition, i.e. anionic [4, 6–8, 10] and cationic [8, 11] liposomes. The net negative charge of the former complicates the nonspecific interaction with cell membrane surfaces, which also possess a net negative charge. Consequently, they may have major opportunities to be taken up as colloidal suspensions via the pinocytic pathway of hepatocytes. For similar reasons, the entrapping of negatively charged DNA into anionic liposomes leads to a low efficiency of encapsulation. Currently, cationic lipids are formulated for gene transfer as a liposome–DNA complex in which DNA is externally associated. The positive lipid charge facilities both liposome–DNA and liposome–cell surface interactions, leading to a relatively efficient entry to the target cell. This type of liposome is the only non-viral vector to have shown sufficient efficacy and safety for the initiation of clinical trials. However, they must be administered locally because the i.v. systemic administration of cationic liposomes produces a wide tissue distribution due to ionic interaction with the outer surface of all cell plasma membranes [11]. With the aim of improving the *in vivo* liver destination of cationic liposomes, the α1-antitrypsin gene has been encapsulated in small liposomes containing 10% of a positively charged lipid (DOTAP) from total lipid vesicle composition, and their *in vivo* transfection efficiency compared with the cationic liposome–DNA complex [8]. The results show that encapsulated DNA is clearly more efficient than the cationic liposome–DNA complex. We believe that it could be due to two main reasons. The first reason is related to hepatocyte targeting ability, since the DNA is encapsulated in small liposomes; in contrast, liposome–DNA complex access to the hepatocyte must be more limited, since it is more heterogeneous in size and has a great diameter. The second reason is related to gene bioavailability, since encapsulated DNA is protected from enzymatic digestion, whereas DNA externally associated to liposome can be digested (at least partially) before the

complex reaches the target cell [12]. How the relative cationic charge of small liposomes entrapping DNA influences their distribution after systemic administration remains an interesting question. Studies of biodistribution and pharmacokinetics are therefore welcome. On the other hand, when the gene transfer efficiencies of anionic and cationic small liposomes were compared, we observed a similar rate of efficiency. Thus, the small liposome size appears to target both anionic and cationic liposomes to liver with a similar efficacy, although we do not know whether both are also using the same pathway for cell internalization. With the aim of clarifying this point, two strategies could be used: modifying the physiological conditions of the liver by a partial hepatectomy and/or preparing liposomes targeted to specific receptors upon the hepatocyte surface, such as asialoglycoprotein receptors. This would then allow us to evaluate the influence of the liposome charge on the expression of a gene delivered by the same receptor-mediated mechanism.

The way by which exogenous genes or nucleic acids enter the cell cytoplasm is a very important step of the gene delivery mechanism of a vector. Cationic lipids also have become important reagents for gene transfer *in vitro* [13, 14], with the ability to provide transfection efficiencies of >90% in some cell lines and primary cells. As the results of ionic interactions between the cationic lipid and anionic DNA, both constitute a particulate complex (from 50 to 700 nm diameter), the efficiency of which for *in vitro* gene deliver can vary by altering the lipid composition and lipid/DNA ratio [14]. Whereas cationic liposome–DNA complex is not a true liposome and nucleic acid is not protected from DNase digestion, conventional liposome formulation, in which compounds are encapsulated within a lamellar lipid structure, protects the DNA from enzymatic digestion and offers advantages for *in vivo* applications. However, comparative studies have demonstrated that such liposomes; are less effective than cationic liposomes in *in vitro* models [15, 16]. Viruses have been selected due to their great ability to infect cells, yielding a very efficient vector to deliver exogenous genes. However, the non-viral vectors lack these selected properties, and the bioavailability of the carried gene is very poor because most of the gene is destroyed within phagolysosomes. Since adenoviral particles mediate endosomal lysis [17] and the co-internalization of exogenous DNA with an adenoviral particle [18, 19] increases (by two or three orders of magnitude) the efficiency of gene delivery, the procedure has become the benchmark against which novel forms of *in vitro* gene delivery are measured and suggests that the inclusion of adenovirus-like properties to non-viral vectors could also increase gene bioavailability by several orders of magnitude.

The efficacy of *in vitro* gene transfer mediated by cationic lipid vesicles has been attributed to the greater uptake of DNA as a result of the facilitated electrostatic interaction with the negative cell surface charge [20]. Therefore, it is interesting to know whether the final charge of conventional liposomes entrapping DNA can influence their efficiency for gene transfer in *in vivo* models. In this respect, the efficiency of α1-antitrypsin gene transfer has been evaluated using positively or negatively charged liposomes. The results suggest that both types of liposomes are able to deliver the exogenous genes with a similar potency [8], since equivalent plasma levels of human protein were observed after the same i.v. dose of recombinant plasmid encapsulated in anionic or cationic liposomes. This observation supports the idea that liposome uptake by cells for which the former exhibit affinity is largely independent of surface charge [21], and that positive liposome surface charge may not be necessary for successful liposome-mediated *in vivo* gene transfection [4, 6, 7, 12, 22]. Early work has shown that neutral or positively charged liposomes attain a net negative surface charge in the presence of blood as a result of plasma protein binding to their surface [23]. Although this observation suggests that a similar affinity for target cells could be expected when using anionic or cationic liposome vectors, the influence of liposome charge on gene delivery efficacy remains to be elucidated. The cellular entrance of small liposomes to *in vivo* hepatocytes could be mediated by a very active pinocytosis mechanism, although anionic and cationic liposomes also could be internalized by different subsets of receptors based on their electrostatic interactions. The influence of liposome charge on gene transfer efficacy, involving the same receptor-mediated liposome-uptake mechanism, is therefore an interesting research topic. Our preliminary results regarding the influence of liposome charge (anionic vs cationic) and of the covalently coupled asialofetium ligand on the liposome surface (asialoglycoprotein receptor-destined liposomes) in mediating human α1-antitrypsin gene transfer to *in vivo* mice (unpublished data) indicated that cationic liposomes are more efficient than anionic liposomes; however, when asialofetuin-liposome was used as the destined vector, anionic liposomes were found to be more efficient than cationic liposomes. The results obtained suggest that signficiant differences could be involved in the process of gene delivery in each case, and support the idea that the ideal vector is likely to be different for each application. At present, the only disadvantage of this hepatocyte-destined approach is the short half-life of the transferred genes. However, it has been described [24] that partial hepatectomy prolongs gene expression of a single dose of a DNA vector, and we are able to confirm that the liposome-mediated expression of human α1-antitrypsin gene in the mouse liver can be prolonged by performing a partial hepatectomy [7, 8]; however, the influence of liposome charge upon gene transfer efficacy, involving receptor-mediated liposome-uptake mechanisms of gene delivery, remains to be elucidated. On the other hand, the prolonged expression of exogenous genes mediated by partial hepatectomy is an unsolved issue, although increased gene bioavailability and/or mechanisms involved in the reactivating of gene expression after cell division could be involved [8, 25–28]. The procedure has a very low potential for clinical application, yet indicates that appropriate gene delivery by

non-viral vectors could lead to the long-term expression of the human α1-antitrypsin gene.

Increased gene bioavailability can be achieved using viral fusogenic proteins to deliver DNA into the cytoplasm of the cell by a liposome-mediated fusion mechanism [29], and encapsulating nuclear proteins to facilitate DNA transport to the nucleus [30, 31]. Since the liposome surface offers opportunities to covalently couple a variety of ligands with different functions (such as target and fusogenic properties), and considering that proteins containing the nuclear localization sequences offer the necessary mechanism for delivering the DNA to the nucleus, we believe that the appropriate combination of these elements may allow the preparation of a liposome vector with the ability to efficiently deliver an expression cassette of interest for clinical applications. This is a very attractive research topic, because it has been estimated that only 0.1% of the plasmid delivered by liposomes actually reaches the nucleus and is expressed. Therefore, the efficiency of liposome-mediated gene transfer could be increased three orders of magnitude if the gene delivery process leads to 100% gene bioavailability.

GENE EXPRESSION CONSTRUCT/GENE INTRINSIC CAPACITY

Exogenous DNA can be integrated into host cell chromosomes using retroviral vectors, but gene delivery employing adenovirus or non-viral gene therapy strategies provides a finite period of gene expression and, therefore, of the therapeutic product. Although spontaneous integration of DNA into genome and homologous recombination has been observed *in vitro* at a low frequency, this event has not been observed *in vivo*. Nevertheless, consideration should be given to the possibility that administered DNA can reside transiently in the nucleus of a target cell as a stable extra-chromosomal (episomal) element where it can be expressed and offer a therapeutic gene product. Obviously, the ultimate efficiency of gene expression is limited by extra- and intracellular events affecting gene bioavailability, but we must consider that the efficacy of gene expression is also limited by the intrinsic capacity of a specific cassette to express the gene product. The selected elements for constructing the expression cassette, such as promoter, enhancer, and stabilizing sequences, greatly influence the gene expression capacity and the stability of the gene product. However, many questions remain to be clarified, including the optimal combination of the elements in every strategy of gene therapy. In many cases, such as α1-antitrypsin deficiency, it is desirable to secure the highest possible level of expression of the transduced gene attainable by a variety of promoters—mainly the cytomegalovirus. This promoter is extremely active in primary hepatocytes [32], although the serum levels of human α1-antitrysin fall after a short period following the hepatocellular transplant of transduced cells [33, 34]. On the other hand, the use of gene constructs under the control of liver-specific promoters [4, 6–8, 35] that are active in normal hepatocytes should be considered in the development of *in vivo* liver-destined gene therapy. In turn, the persistence of genes within the liver and the duration of gene product expression can be prolonged by the *in vivo* stable integration of transferred genes mediated by retroviral vectors [35]; moreover, the expression of exogenous genes delivered by non-viral vectors can be prolonged by performing a partial hepatectomy [7, 8, 24]. As the latter is difficult to apply within the clinical context, further work is required to combine the liver-destined non-viral vector approaches with mechanisms for the stable establishment of the therapeutic gene within the liver. In this sense, and based on previous observations [35], we could speculate that sequences corresponding to the replication origin from Epstein–Barr virus could contribute to maintaining the exogenous gene as episomal DNA for longer periods of time in resting hepatocytes. However, it is necessary to emphasize that in many cases optimal gene therapy should be based on gene repair and/or the introduction of a normal gene by homologous recombination, although this constitutes an as yet unsolved problem in liver-destined *in vivo* gene therapy.

We thank Dr. David T. Curiel (Comprehensive Cancer Center, Gene Therapy Program, University of Alabama at Birmingham) for his valuable revision of the manuscript. This work has been supported in part, by CICYT PB92-0877 and CICYT SAF96-0229 projects.

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