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Translational Challenges for Hepatocyte-Directed Gene Transfer

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1. Introduction

The liver is a central organ in many metabolic processes. Multiple inherited metabolic disorders have their origin in this organ. Therefore, hepatocytes are a key target for gene therapy directed at correction of inborn errors of metabolism and of hemophilia. Inborn errors of metabolism lead to accumulation of toxic products in hepatocytes and extensive hepatotoxicity, as observed in disorders like α_1-antitrypsin deficiency, type I tyrosinemia, or Wilson disease[1]. In other metabolic disorders, such as in Crigler-Najjar syndrome type I, ornithine transcarbamylase deficiency, familial hypercholesterolemia, and hemophilia A and B, manifestations are primarily extrahepatic[1]. In addition, the liver is a target for gene therapy of acquired diseases such as liver cancer and hepatitis[2, 3].

Insights into the determinants of gene transfer efficiency to hepatocytes are therefore required to evaluate the potential of gene therapy for inborn errors of metabolism and for acquired liver diseases. These determinants include innate and adaptive immune responses, cellular and biochemical determinants of hepatocyte transduction such as ligand receptor interactions, and anatomical and histological factors. Here we will first focus on the role of liver sinusoidal cells and sinusoidal fenestrae as determinants of the efficiency in hepatocyte-directed gene transfer. Uptake of gene transfer vectors by Kupffer cells and liver sinusoidal endothelial cells limits hepatocyte transduction[4-11]. However, the presence of fenestrae in liver sinusoidal endothelial cells provides a direct access to the space of Disse and microvillous surface of hepatocytes and may allow transcellular migration of vectors. In the following paragraphs, we provide substantial evidence that the interplay between these opposing forces, i.e. uptake by non-parenchymal liver cells and transcellular migration through fenestrae, is a critical determinant of gene transfer efficiency into hepatocytes.

Before discussing the different parameters that influence the efficiency of hepatocyte-directed gene transfer, it is important to point out that the process of gene transfer to parenchymal liver cells using replication-defective virus-derived vectors or non-viral vectors is fundamentally different from infection of hepatocytes by hepatotropic viruses. After successful infection, viruses replicate, leading to an exponential local increase of viral load. Consequently, a very small inoculum may be sufficient to induce hepatitis. In contrast, the number of transgenes carried by viral gene transfer vectors that pass through fenestrae cannot be amplified in hepatocytes since these vectors are replication defective. The number of vector particles that pass through fenestrae and are subsequently taken up by hepatocytes is therefore a critical determinant of transgene DNA copy number in these cells and of transgene expression levels.

2. Liver anatomy and histology

The liver is the largest internal organ of the human body, weighs about 1.5 kg, and is located in the upper right quadrant of the abdomen. The liver is highly vascularised and the total blood flow through the liver can reach up to 25% of the cardiac output. Approximately 75% of the blood that enters the liver derives from the portal vein that carries oxygen-poor blood. Oxygenated blood entering the liver via the hepatic artery constitutes the remaining 25% of blood supply. The portal vein, the hepatic artery, lymphatic vessels, nerves, and bile ducts enter the liver at the hilus. From the hilus, continuous branching of the hepatic artery and portal vein results in an intricate network of intertwining capillaries, called sinusoids. Histologically, the liver is divided into lobuli, hexagonal functional units formed by hepatocytes and sinusoids surrounding a central vein. Neighbouring lobules are surrounded by portal triads, consisting of branches of the bile duct, the portal vein, and the hepatic artery.

A human liver comprises 4.5×10^{11} cells[12, 13]. A murine liver contains approximately 1.6×10^{8} cells[12] whereas a rabbit liver counts about 1.5×10^{10} cells. These data allow to calculate the ratio of transgene copies contained in a given vector dose versus the number of hepatocytes, which can be calculated as 66% of the total number of liver cells. Empirical determination of the transgene DNA copy number per hepatocyte is hampered by the fact that copy numbers per diploid genome do not accurately reflect transgene DNA copy numbers per hepatocyte. Indeed, hepatocytes are frequently tetraploid or even octoploid and may also have a binuclear nucleus.

3. Liver sinusoidal cells

Sinusoidal cells constitute approximately 33% of the number of resident liver cells whereas parenchymal liver cells or hepatocytes comprise the remaining cells[14-16]. Sinusoidal cells are a compilation of endothelial cells, Kupffer cells (resident liver macrophages), fat-storing cells (also called stellate cells or Ito cells), and pit cells (natural killer cells). Liver sinusoidal endothelial cells comprise 70%, Kupffer cells 20%, stellate cells 10%, and pit cells less than 1% of the number of sinusoidal cells[14, 16, 17]. In the context of hepatocyte-directed gene transfer, we focus here on the role of Kupffer cells and liver sinusoidal endothelial cells that constitute together the reticulo-endothelial cells of the liver. Kupffer cells account for 80% to 90% of resident macrophages in the entire body[18]. They have a diameter of 10-15 μm whereas liver sinusoidal endothelial cells are smaller with a diameter of 7-9 μm[14]. Sinusoidal endothelial cells are scavenger cells that are able to internalize particles up to 0.23 μm under physiologic conditions *in vivo*[19]. Larger particles are taken up by Kupffer cells[19]. Since most gene transfer vectors have a diameter below 0.23 μm, uptake of vectors by both Kupffer cells and liver sinusoidal endothelial cells may attenuate the efficiency of hepatocyte-directed gene transfer.

Most experimental work on the role of liver reticulo-endothelial cells in relation to hepatocyte transduction has been performed with adenoviral vectors. These studies support the dual role of liver reticulo-endothelial cells in hepatocyte-directed gene transfer. On the one hand, the high endocytotic capacity of these cells limits hepatocyte transduction, on the other hand, the presence of fenestrae facilitates hepatocyte transduction[20]. The relevance of these observations for other types of vectors and other modes of gene transfer will subsequently be discussed.

4. In vitro and *in vivo* transduction by adenoviral vectors

Efficient hepatocyte transduction by adenoviral vectors requires that two clearly distinct conditions are met. First, adenoviral vectors should have a facilitated access to the space of Disse via sufficiently large sinusoidal fenestrae. Second, vectors in the space of Disse must be able to bind to cellular receptors on hepatocytes for internalisation and transduction. Thus, both the anatomical access of vectors to the space of Disse and the potential for interaction with hepatocyte receptors *in vivo* are necessary but not always sufficient for hepatocyte transduction *in vivo*. Before going into the anatomical access of vectors to the space of Disse, we will first discuss differences between adenoviral transduction *in vitro* and transduction of hepatocytes *in vivo*.

For humans, 51 adenovirus serotypes have been identified, and these serotypes are classified into 6 species (A-F). *In vitro*, uptake of most Ad serotypes belonging to species A, C, D, E, and F, is initiated by binding of the adenovirus fiber protein to coxsackie and adenovirus receptor (CAR) on the cell surface[21, 22]. CD46, a complement regulatory protein that is ubiquitously expressed in humans[23], but only in the testis in mice[24], is a cellular receptor for group B adenoviruses[23]. Human species C adenovirus serotype 5 (Ad5) is the most common viral vector used in clinical studies worldwide[25]. *In vitro*, Ad5 infects cells through binding of the fiber to CAR, followed by binding of an arginine-glycine-aspartic acid (RGD)-motif on the Ad5 penton base with cellular integrins (mainly $\alpha_v\beta_3$ and $\alpha_v\beta_5$) which initiates receptor-mediated endocytosis via clathrin coated pits[26, 27]. However, CAR binding ablation[28, 29] and α_v integrin binding ablation[29, 30] do not significantly reduce liver transduction by adenoviral vectors *in vivo*. The direct Ad binding to hepatic heparan sulfate proteoglycans via the KKTK motif within the fiber shaft domain has been suggested to be the major mechanism of hepatocyte transduction *in vivo*[31, 32]. However, mutation of the KKTK motif in the Ad5 fiber shaft renders the fiber inflexible and prevents internalisation of Ad5 though steric hindrance[33, 34]. *In vitro* and *in vivo* infectivity studies of Ad5-based vectors possessing long Ad31- (species A) or Ad41- (species F) derived fiber shaft domains that lack the KKTK motif, have shown that these vectors transduce hepatocytes with similar efficiency compared to Ad5 vectors[35], consistent with the absence of a critical role of the KKTK motif in hepatocyte transduction.

An important difference between *in vitro* transduction and *in vivo* transduction of liver cells after intravenous injection is that adenoviral vectors are in contact with blood proteins. Treatment of mice with the vitamin K antagonist warfarin, which inactivates several proteins of the coagulation cascade (factor II, factor VII, factor IX, factor X) as well as the anticoagulant protein C, abrogates transduction of hepatocytes by adenoviral serotype 5 vectors[8, 36-38]. These studies suggested that a coagulation protein or coagulation proteins have a bridging function in the entry of liver cells by adenoviral vectors. Only factor X could rescue liver transduction in warfarin anticoagulated mice[39]. Recently, it has been shown that the γ-carboxyglutamic acid domain of factor X binds in a calcium-dependent manner to hexon protein in adenovirus serotype 5[25, 39, 40]. Factor X binds at the cup formed by the center of each hexon trimer. Serotypes with a high affinity for factor X, such as the species C serotypes Ad2 and Ad5, have been shown to efficiently transduce hepatocytes following intravenous administration[41, 42]. In contrast, species B Ad35 and species D Ad26 bind to factor X weakly or not at all, and fail to transduce hepatocytes[39, 43, 44]. More specifically, factor X binds to the adenovirus hexon hypervariable regions (HVRs). Liver infection by the factor X-Ad5 complex is mediated through a heparin-binding exosite in the factor X serine

protease domain. Substitution of HVR5 or HVR7 from Ad5 with sequences from the non-factor X binding serotype Ad26 substantially lowered factor X binding and liver transduction *in vivo*[45]. An Ad5 mutant containing an insertion in HVR5 was shown to bind factor X *in vitro* with 10 000-fold reduced affinity compared with unmodified vector and failed to deliver the red fluorescent protein transgene *in vivo*[25]. Taken together, factor X binding to hexon trimer is a necessary prerequisite for hepatocyte transduction *in vivo*[39].

5. Liver trapping of adenoviral vectors

Previous studies have shown that different adenoviral serotypes are rapidly sequestered in the liver after intravenous delivery, independent of their potential to effectively transduce hepatocytes[43, 46, 47]. Trapping of adenoviral vectors in the liver is comparable between wild-type mice and mice treated with warfarin, which shows that factor X-facilitated adenoviral vector entry into hepatocytes is not required for trapping of vectors in the liver[8]. Demonstration of liver sequestration using whole livers does not make a distinction between the presence of vectors extracellularly (in the vascular lumen of the sinusoids or in the space of Disse) or intracellularly (in the non-parenchymal liver cells of the parenchymal liver cells). Cellular uptake of adenoviral vectors after systemic gene transfer occurs predominantly in non-parenchymal liver cells (i.e. mainly liver sinusoidal endothelial cells and Kupffer cells)[4-7]. Kupffer cells may bind adenoviral vectors via multiple mechanisms including scavenger receptor-A, complement, and natural antibodies[9-11] and this uptake is independent of factor X[10, 11]. Interactions of adenoviral vectors with platelets in blood may contribute significantly to sequestration in the reticuloendothelial system of the liver[9]. Nevertheless, the exact mechanisms of adenoviral vector uptake in Kupffer cells have not been elucidated. The amount of Ad vector DNA after intravenous administration was nearly identical in wild-type mice and scavenger receptor-A deficient mice[11] consistent with the presence of multiple pathways leading to Kupffer cell sequestration[10].

Recently, Di Paolo *et al*.[11] showed that simultaneous treatment of mice with warfarin and clodronate liposomes, that deplete Kupffer cells, results in only a minor reduction of sequestration of adenoviral vectors in the liver 1 hour after gene transfer. Transmission electron microsopy showed the presence of vectors in the space of Disse, consistent with anatomical sequestration of vectors. We suggest that the presence of fenestrae is crucial in the liver targeting of adenoviral vectors. In other words, the targeting of adenoviral vectors to the liver reflects predominantly or exclusively anatomical targeting. This also implies that molecular strategies directed at liver detargeting of adenoviral vectors should take into account the existence of anatomical targeting to the liver.

6. Uptake of gene transfer vectors by reticulo-endothelial cells of the liver reduces hepatocyte transduction

Both Kupffer cells and liver sinusoidal endothelial cells take up the large majority of adenoviral vectors after systemic gene transfer[7]. Uptake of vectors by non-parenchymal liver cells (i.e. mainly liver sinusoidal endothelial cells and Kupffer cells) inversely correlates with transduction of parenchymal liver cells[7]. The transgene DNA copy number in the non-parenchymal liver cells at one hour after transfer in Balb/c mice was nearly 6-fold higher than in C57BL/6 mice[7]. This difference in scavenging of vectors between both strains is a major determinant of the approximately 3-fold higher transgene DNA levels in hepatocytes

and higher transgene expression levels in C57BL/6 mice compared to Balb/c mice[7]. Based on more refined experiments with isolation of Kupffer cells and liver sinusoidal endothelial cells, we showed that the transgene DNA copy number per diploid genome at 1 hour after transfer in C57BL/6 mice was 2.9-fold higher in liver sinusoidal endothelial cells than in Kupffer cells[7]. In contrast, the copy number in Kupffer cells was 2.6-fold higher than in liver sinusoidal endothelial cells in Balb/c mice. These data indicate that the relative contribution of liver sinusoidal endothelial cells and Kupffer cells to adenoviral vector clearance may be highly dependent on the specific genetic context. One explanation for this difference of uptake of adenoviral vectors by the liver reticulo-endothelial cells of C57BL/6 and Balb/c mice may be the differential modulation of the function of these cells by humoral factors produced by spleen cells. Indeed, a significantly reduced transgene DNA copy number was observed in the liver reticulo-endothelial cells one hour after adenoviral transfer in splenectomized Balb/c mice and in Balb/c rag-2$^{-/-}$ mice compared to control Balb/c mice[7]. This was accompanied by a significantly higher transgene DNA copy number in hepatocytes of splenectomized Balb/c mice and of Balb/c rag-2$^{-/-}$ mice than in hepatocytes of wild-type Balb/c mice[7]. Splenectomy in Balb/c rag-2$^{-/-}$ mice did not result in an incremental effect[7]. This suggests that humoral factors produced by spleen lymphocytes may affect the clearance of adenoviral vectors by liver reticulo-endothelial cells in Balb/c mice. In contrast, no such effects on intrahepatic transgene DNA distribution were observed in splenectomized C57BL/6 mice and in C57BL/6 rag-1$^{-/-}$ mice, suggesting highly heterogeneous effects of humoral factors produced by spleen lymphocytes on liver reticulo-endothelial cells.

Further evidence for a major role of liver reticulo-endothelial cells as a determinant of hepatocyte transduction comes from experiments with clodronate liposomes. Depletion of Kupffer cells and macrophages in the spleen by intravenous administration of clodronate liposomes results in significantly increased transgene DNA levels in parenchymal liver cells[7] and in increased transgene expression[6, 7, 48, 49]. Since liver sinusoidal endothelial cell function may be modified by Kupffer cells[50, 51], it cannot be excluded that part of the effect of clodronate liposomes is due to reduced activation of liver sinusoidal endothelial cells by Kupffer cells. Besides clodronate liposomes, pre-administration of polyinosinic acid, a scavenger receptor A ligand, before gene transfer has been shown to prevent sequestration of adenoviral vectors in Kupffer cells and to enhance parenchymal liver cell transduction[52]. Transient saturation of the reticulo-endothelial system with phosphatidylcholine liposomes or with Intralipid® also reduces uptake of vectors in the non-parenchymal liver cells and augments hepatocyte transduction[7]. Taken together, various interventions that result in reduced uptake of adenoviral vectors in liver reticulo-endothelial cells consistently enhance hepatocyte transduction.

7. Liver sinusoidal endothelial fenestrae

Liver sinusoids are highly specialized capillaries with two critical features: the thin endothelium contains open fenestrae, whereas a basal lamina is lacking[53]. Fenestrae are clustered in sieve plates and provide an open pathway between the sinusoidal lumen and the space of Disse, in which numerous microvilli from parenchymal liver cells protrude[53, 54]. Sinusoidal fenestrae have no diaphragm and visualisation requires perfusion fixation with glutaraldehyde. Scanning electron microscopy analysis has shown that sinusoidal fenestrae

comprise 6–8% of the sinusoidal surface[55]. Compared to the centrilobular area, the diameter of fenestrae is larger but the frequency of fenestrae is lower in the periportal area[55, 56].
The open communication between the sinusoidal lumen and the space of Disse through fenestrae represents a unique route that provides direct access for gene transfer vectors to the surface of hepatocytes. However, fenestrae act as a sieve and will mechanically restrict the transport of gene transfer vectors according to their size. Thus, two parameters must be taken into account when considering the access of gene transfer vectors to hepatocytes namely the diameter of both fenestrae and gene transfer vectors.

8. Species variation of the average diameter of fenestrae

Fenestrae generally measure between 100 nm and 200 nm and significant species differences in their size exist[53-55]. However, the interpretation of the existing literature on species variations of the size of sinusoidal fenestrae is hampered by differences in preparatory methods applied by different investigators. Standardised protocols within one group of investigators are therefore *a conditio sine qua non* for reliable species and strain comparisons. A direct comparative study of the diameter of sinusoidal fenestrae in five species using scanning electron microscopy was performed by Higashi *et al*.[57]. The average diameter of sinusoidal endothelial fenestrae in this study was 45 nm in cows, 52 nm in sheep, 66 nm in guinea pigs, 82 nm in pigs, and 131 nm in dogs[57]. However, these results are based on scanning electron microscopy preparations and are therefore subject to a shrinkage effect in the order of 30% caused by dehydration and drying of the tissue[58]. Accurate measurements of fenestrae can only be obtained by gradually replacing cellular water by plastic during preparation for transmission electron microscopy[59]. Previous studies have shown that this method of preparation leads to accurate measurements of cellular details, such as fenestrae. Visualisation of fenestrae in transmission electron microscopy sections requires that endothelial cells and their sieve plates are cut tangentially so that fenestrae become visible as holes. Using this technology and standardized protocols, it has previously been shown that the average diameter of fenestrae is significantly larger in Sprague Dawley rats (150 nm in the pericentral area and 175 nm in the periportal area)[55] and C57BL/6 mice (141 nm)[60] than in New Zealand White rabbits (103 nm)[60], Fauve de Bourgogne rabbits (105 nm)[61], and humans with a healthy liver (107 nm)[62]. The diameter in Dutch Belt rabbits was intermediate (124 nm)[61]. Taken together, this species comparison demonstrates that the diameter of fenestrae in humans is similar to New Zealand White rabbits and significantly smaller compared to mice and rats, two species that are most frequently used in gene transfer studies. The occurrence of major shrinkage effects in scanning electron microscopy samples is indicated by the significantly lower diameters reported for New Zealand White rabbits (49 nm)[63] and Sprague Dawley rats (89 nm)[63] as compared to diameters obtained in transmission electron microscopy studies[55, 60]. Transmission electron microscopy studies consistently show that the interindividual variation of the average diameter of fenestrae within the same species or strain is low, as indicated by coefficients of variation between 3-8%. In contrast, as will be discussed in the next paragraph, the intraindividual variation of diameters of fenestrae is high.

9. Intraindividual variation of the diameter of fenestrae

The intraindividual variation of the diameter of fenestrae is an important parameter that may complicate investigations on the relation between the diameter of fenestrae and gene

transfer efficiency to hepatocytes. The distribution of the diameters of fenestrae within one individual or within one animal follows a Gaussian function with some skewing to the right[20].

Two opposing processes will determine the entrance of vectors into the space of Disse: on the one hand passage through sinusoidal fenestrae and on the other hand endocytosis by Kupffer cells and endothelial cells. Species and strain differences in transendothelial passage will be determined by intrinsic differences of the function of liver reticulo-endothelial cells (e.g. C57BL/6 versus Balb/c mice) as well as the rate passage of vectors through fenestrae. Based on these considerations, one can predict that the ratio of transgene DNA copy number in parenchymal liver cells versus the copy number in sinusoidal liver cells will correlate positively with the diameter of fenestrae. After reviewing data on the diameters of different gene transfer vectors, we will present several lines of experimental evidence that support the critical role of the diameter of fenestrae in hepatocyte transduction.

10. Diameters of gene transfer vectors

To put the importance of the size of fenestrae for hepatocyte-directed gene transfer into perspective, accurate knowledge of the diameter of gene transfer vectors is required. To avoid bias in the measurement of the diameter of adenoviral vectors, we previously vitrified a sample of adenoviral vectors using Vitrobot™ technology and determined the diameter by cryo-electron microscopy[60]. Adenoviral serotype 5 virions were shown to have a diameter of 93 nm with protruding fibers of 30 nm[60]. Using the same imaging techniques, the diameter of a vesicular stomatitis virus-G pseudotyped human immunodeficiency virus-1 derived lentiviral vector was found to be 150 nm[60]. Adeno-associated viral serotype 2 vectors have an average diameter of 22 nm[64]. Herpes simplex virions have been reported to be as large as 180 nm[65]. The diameter of liposomes used for non-viral gene transfer varies between 50 nm and 1000 nm and is highly dependent on production parameters[66, 67].

11. Experimental evidence for a critical role of sinusoidal fenestrae in hepatocyte transduction following adenoviral gene transfer

Based on our prior studies in different strains of rabbits and in different species[60-62], the correlation coefficient between the average diameter of sinusoidal fenestrae in these different strains and species and human apo A-I expression at day 7 after transfer with an adenoviral vector containing a hepatocyte-specific expression cassette was found to be 0.94 ($p<0.01$). This strongly suggests that the diameter of sinusoidal fenestrae is an important determinant of gene transfer efficiency to hepatocytes.

To demonstrate that the difference of human apo A-I plasma levels reflects differences of transgene DNA levels in parenchymal liver cells, we isolated parenchymal and non-parenchymal liver cells at day 3 after transfer in C57BL/6 mice and New Zealand White rabbits. Transgene DNA levels in parenchymal liver cells were much higher in C57BL/6 mice than in New Zealand White rabbits whereas the reverse pattern was observed in non-parenchymal liver cells[60]. Considering the small average diameter of fenestrae in New Zealand White rabbits (103 nm), it appears that the sinusoidal wall constitutes a histological barrier for adenoviral vectors in this species leading to increased uptake by liver reticulo-endothelial cells. In contrast, the larger fenestrae in C57BL/6 mice (141 nm) facilitate access to hepatocytes, leading to increased uptake into hepatocytes and to reduced scavenging by

Kupffer cell and liver sinusoidal endothelial cells. In other words, the size of fenestrae determines the distribution of vectors between sinusoidal and parenchymal liver cells.

Although the relation between the diameter of sinusoidal fenestrae and transgene expression after adenoviral gene transfer may be confounded by substantial differences in genetic background, we showed that interventions that increase the diameter of fenestrae result in New Zealand White rabbits significantly increased transgene expression[60, 61]. These two intervention studies support the view that the correlation between the diameter of fenestrae and transgene expression after adenoviral transfer reflects a causal relationship.

Based on the high degree of similarity of the distribution of the diameter of fenestrae between humans and New Zealand White rabbits[20, 68], one would predict a low efficiency of gene transfer into hepatocytes after adenoviral transfer in humans. In the ornithine transcarbamylase deficiency trial, low levels of gene transfer in hepatocytes were indeed observed[69]. The authors concluded that the level of transgene expression was lower than what would have been predicted based on preclinical animal models[69]. Although histological alterations of the livers in patients with partial ornithine transcarbamylase deficiency may have contributed to low hepatocyte transduction, we speculate that a much smaller size of fenestrae in humans compared to mice and rats is likely the most critical factor in the observed species difference of hepatocyte transduction. On the other hand, the small diameter of fenestrae in humans may be beneficial for the efficacy of molecular strategies directed at liver detargeting of adenoviral vectors since anatomical targeting to the liver will be limited.

Recently, Brunetti-Pierri *et al.*[70] developed a minimally invasive procedure that significantly improves the efficiency of hepatocyte-directed transfer in nonhuman primates. A balloon occlusion catheter was percutaneously positioned in the inferior *vena cava* to occlude hepatic venous outflow[70]. Gene transfer of gutted vectors was performed via a percutaneously placed hepatic artery catheter with an infusion time of 7.5 minutes or 15 minutes. This procedure resulted in approximately 10-fold higher transgene expression levels compared to systemic gene transfer. Increased intrahepatic pressure following occlusion of hepatic outflow of the liver may increase the diameter of fenestrae, similar as observed following hydrodynamic injections in mice[71], and this may contribute to the beneficial effects of this procedure in monkeys.

12. Potential relevance of sinusoidal fenestrae for other modes of hepatocyte-directed gene transfer

Based on the data obtained with adenoviral vectors, it is likely that the large diameter of lentiviral vectors is an important limitation for hepatocyte-directed gene transfer and may restrict passage of vectors even in mice and rats. Indeed, gene transfer efficiency in mice and rats is low after *in vivo* lentiviral gene transfer[72-75]. Although other factors like technological challenges to obtain high titer vector stocks may play a role, it is likely that the large diameter of lentiviral vectors is a limitation for hepatocyte-directed gene transfer. Since this anatomical limitation does not exist for adeno-associated viral vectors, gene transfer efficiency into hepatocytes with this type of vectors will be solely dependent on cellular and molecular determinants of hepatocyte transduction.

Fenestrae may also play a role in naked DNA transfer. Liu *et al.*[76] showed that the murine liver can rapidly extract up to 25 μg of plasmid DNA from the blood during a single pass after simple intravenous injection. Moreover, this study showed that naked DNA is

primarily taken up by the liver endothelial cells, but not by Kupffer cells, and that transfection of hepatocytes can be improved by mechanical massage of the liver, which increases the size of liver sinusoidal fenestrae[76]. Substantial amounts of plasmid DNA are degraded by nucleases in the blood following simple intravenous injection, which can be overcome by hydrodynamic gene transfer. It has also been proposed that fenestrae play a role in the transport of naked DNA into hepatocytes during hydrodynamic gene transfer[71]. Although the exact mechanism of hepatocyte transfection following hydrodynamic gene transfer remains to be elucidated, a general consensus is that the injected volume induces right heart volume overload. This results in a retrograde flow through the *vena cava* and in particular in a retrograde flow into the hepatic veins. As a result, intrahepatic pressure increases and the DNA containing solution is forced out of the hepatic sinusoids into the parenchymal liver cells. Following systemic hydrodynamic gene transfer in mice and rats, the majority of the injected DNA (i.e. >90%) can be retrieved in the liver[77]. In addition, microscopic analysis has indicated that transfected hepatocytes are predominantly located in the pericentral region[78]. This predilection may be explained by the fact that sinusoids are wider and straighter and contain more fenestrae per unit of surface in the pericentral area than in the periportal area[56, 77].

13. Sinusoidal fenestrae and hepatocyte transduction in diseased livers

The unique morphological features of liver sinusoidal endothelial cells may change in pathological conditions. Liver fibrosis and cirrhosis lead to a decreased number of fenestrae[79] and capillarization and perisinusoidal fibrosis leads to the development of a basal lamina, found to be absent in normal sinusoids. A significant reduction in the number of fenestrae and porosity of the sinusoidal endothelial cells was observed in alcoholic liver disease without cirrhosis[80]. In a comparative study, decreased transduction by adenoviral vectors has been observed in cirrhotic rat livers compared to normal livers[81]. Furthermore, hydrodynamic gene transfer was significantly less efficient in rats with a fibrotic liver compared to rats with a healthy liver[82]. Sinusoidal capillarization also occurs in hepatocellular carcinoma[83, 84]. This may constitute a major obstacle for efficient gene therapy for liver cancers.

14. General perspective

Preclinical viral and non-viral gene transfer studies should consider scavenging of vectors by liver reticulo-endothelial cells and as well as the diameter of sinusoidal fenestrae as important determinants of gene transfer efficiency into hepatocytes. Although the diameter of fenestrae may be modulated to some extent, there is currently no safe pharmacological intervention that results in a significant enlargement of fenestrae. The small diameter of fenestrae in humans and alterations of liver sinusoidal endothelial cells in liver disease may constitute a significant and potentially insurmountable obstacle for efficient gene transfer into hepatocytes with several vectors. Both anatomical access of vectors to the space of Disse on the one hand and the potential of vectors for interaction with hepatocyte receptors *in vivo* on the other hand are necessary for efficient hepatocyte transduction *in vivo*. A model on hepatocyte transduction should therefore take into account that both an anatomical prerequisite and a molecular prerequisite have to be met.

15. Innate and adaptive immune responses: an introductory perspective

The use of non-viral gene therapy vectors, including naked DNA and liposomes, results in very low to suboptimal expression levels with the possible exception of hydrodynamic gene transfer[85-87]. However, this latter method is only successful in mice and rats and attempts for implementation of this methodology in larger species have resulted in very low gene transfer efficiencies[88-90]. Therefore, our strategic point of view is that only viral vectors constitute sufficiently potent gene delivery platforms to treat genetic and acquired diseases. Although viral vectors are non-replicative in contrast to wild-type viruses, the immune system is efficient to fight off what it perceives as invading pathogens. Innate immune responses are initiated by recognition of pathogen-associated molecular patters by pattern recognition receptors like Toll-like receptors on the surface of professional antigen presenting cells. Subsequent production of inflammatory cytokines stimulates maturation of antigen presenting cells, enhances their endocytic activity, and upregulates expression of molecules required for antigen processing and presentation and for costimulation. Viral capsid proteins not only elicit innate immunity but are also viable targets for host adaptive immune responses that do not necessarily require *de novo* viral gene expression. Adaptive immune responses against vector-derived antigens may reduce the efficacy of *in vivo* gene transfer and may prevent readministration. Furthermore, many vectors are derived from parent viruses that humans have encountered through natural infection, resulting in pre-existing antibodies and possibly in memory responses against vector antigens. Taken together, adaptive immune responses represent one the most challenging remaining hurdles for the development of viral hepatocyte-directed gene transfer strategies with a sufficient therapeutic index. Besides the issue of adaptive immune responses against the vector and the potential problem of pre-existing immunity, immune responses against the transgene product also constitute a hurdle.

16. Innate immune responses after viral gene transfer

Adenoviral vectors efficiently transduce liver cells after systemic gene transfer and expression levels are generally significantly higher as compared to non-viral, adeno-associated viral, and lentiviral transfer. However, recognition of molecular patterns on adenoviral capsids by pattern recognition receptors on macrophages and dendritic cells triggers innate immune responses and induces the production of several cytokines and chemokines[6, 91-96]. Severe activation of the innate immune system, as observed in patients with systemic microbial infections, severe trauma, or after major surgery, may lead to a systemic inflammatory response syndrome, or even to multiple system organ failure and shock. The development of systemic inflammatory response syndrome and multiple system organ failure after adenoviral transfer is dose and species dependent[69, 94, 97]. Schnell *et al.*[94] demonstrated significant species variation in innate immune responses after adenoviral gene transfer. Mice did not develop clinical symptoms of systemic inflammatory response syndrome at any dose of vector whereas rhesus monkeys developed liver necrosis and coagulopathy at a dose of 10^{13} particles/kg of an E1-deleted vector[94]. The sensitivity of humans to adverse effects of innate immune responses after adenoviral transfer is significantly higher than in rhesus monkeys as significant side-effects have been observed at doses of 6×10^{11} particles/kg or even lower[69].

Interleukin (IL)-6 is not only produced by macrophages and T cells but also by liver sinusoidal endothelial cells[98] and possibly by spleen sinusoidal endothelial cells.

Notwithstanding the fact that Kupffer cells are by large the most numerous population of resident macrophages in the body, we have shown that the predominant source of IL-6 after adenoviral transfer in C57BL/6 mice is the spleen and not the liver or lungs[99]. This finding is based both on gene transfer experiments in splenectomized mice as well as on quantification of IL-6 mRNA levels in different organs following adenoviral transfer[99].

Innate immune responses following adenoviral transfer are dependent on both Toll like receptor (TLR) 2 and TLR9[100]. TLR2 and TLR9 are expressed in Kupffer cells but also in the spleen[101-103] as well as in liver sinusoidal endothelial cells[102].

Depletion of tissue macrophages and dendritic cell subpopulations in liver and spleen by the administration of liposomes encapsulating dichloromethylene-biphosphonate results in reduced plasma levels of IL-6, IL-12, and tumor necrosis factor (TNF)-α[93]. However, this cytotoxic strategy is unlikely to be applicable in humans. Conjugation of adenoviral vectors with activated monomethoxypolyethylene glycols (MPEG) has been shown to reduce IL-6 plasma levels after adenoviral transfer[99, 104]. In addition, we have shown that the combination of PEGylation of adenoviral vectors and administration of methylprednisolone completely suppresses elevations of IL-6 levels after transfer with E1E3E4-deleted adenoviral vectors at a dose of 4×10^{12} particles/kg[99]. This combined strategy also inhibits chemokine expression in the liver, abrogates neutrophil infiltration and T-lymphocyte infiltration in the liver, and reduces elevations of serum transaminases in the early phase after adenoviral transfer[99].

Since dose reduction represents an additional means to attenuate innate immune responses against adenoviral vectors, strategies to enhance the efficiency of hepatocyte transduction are required to obtain therapeutic expression levels at lower doses. Brunetti-Pierri *et al*.[70] developed a minimally invasive procedure that significantly improves the therapeutic index of hepatocyte-directed transfer in nonhuman primates. A balloon occlusion catheter was percutaneously positioned in the inferior *vena cava* to occlude hepatic venous outflow[70]. Gene transfer of gutted vectors was performed via a percutaneously placed hepatic artery catheter with an infusion time of 7.5 minutes or 15 minutes. This procedure resulted in approximately 10-fold higher transgene expression levels compared to systemic gene transfer. At vector doses of 1×10^{10} or 3×10^{10} particles/kg, IL-6 levels were consistently below 100 pg/ml[70].

In contrast to adenoviral gene transfer, adeno-associated viral gene transfer induces very weak or absent innate immune responses to viral capsids both in mice[95] and monkeys[105]. Serum levels of five major inflammatory cytokines (TNF-α, interferon-γ, IL-6, IL-10, and IL-12) were not elevated in macaques after gene transfer with vectors based on adeno-associated virus serotypes 2, 7, and 8[105]. These data suggest that very weak or absent innate immune responses to adeno-associated viral capsids may be a general observation in different species. Nevertheless, some level of innate immune activation occurs even with adeno-associated viral vectors[106].

17. Adaptive immune responses after hepatocyte-directed gene transfer

T helper cell activation is complex. Besides signalling via pattern recognition receptors (signal 0), T cell activation requires interaction of the T cell receptor CD3 complex with antigen presented in Major Histocompatibility Complex II (MHCII) (signal 1), engagement of costimulatory molecules such as CD80/86 on antigen presenting cells with CD28 on T cells or CD40 on antigen presenting cells with CD40 ligand on T cells (signal 2), and a

specific cytokine milieu (signal 3) that primes a T helper 1 (Th1) or a T helper 2 (Th2) response. Immunological tolerance is a state in which the immune system is not capable to activate the appropriate cellular or humoral immune responses following antigen exposure. In absence of signal 1, naive T cells are never primed, resulting in immunological ignorance. In the absence of adequate costimulation (signal 2), immunological tolerance is based on anergy/deletion: antigen-specific T cells are primed but are functionally deficient with regard to proliferation and cytokine production. In the absence of signal 3, regulatory T cells (Tregs) may develop. Tregs actively suppress adaptive immune effectors. Taken together, there are multiple requirements for adequate priming of T cells, which offers perspectives for induction of antigen specific tolerance.

Immune responses in mice are highly strain dependent. C57BL/6 mice preferentially develop a predominantly Th1-type immune response whereas Balb/c mice develop a Th2-type immune response[107]. Nevertheless, we have shown that cellular immune responses against adenoviral epitopes do not play a role in transgene DNA kinetics in C57BL/6 mice[108]. This implies that, in the absence of an adaptive immune response against the transgene product, non-immune mechanisms are responsible for the decrease of transgene DNA over time. Whether this is also the case in Balb/c mice, is unknown. Taken together, episomal stability of non-integrated transgenes is an important issue. This is further highlighted by our observations that the persistence of transgene DNA is dependent on the expression cassette design[109-111].

Treatment of genetic diseases by gene replacement therapy is hampered by adaptive immune responses against the transgene product. The risk of antibody formation against the transgene product may be limited in the specific setting of hepatocyte-directed gene transfer[112-114] and specifically by restricting transgene expression to hepatocytes by use of hepatocyte-specific expression cassettes[115, 116].

We consistently observed the absence of antibodies against human apolipoprotein (apo) A-I in all investigated murine strains after gene transfer with vectors containing a hepatocyte-specific expression cassette[110, 115]. This absence of a humoral immune response has also been observed after gene transfer with the same vector in rats[117]. However, this observation cannot be robustly extrapolated to other species. In contrast to findings in mice and rats, we have previously observed a vigorous humoral immune response against human apo A-I in three different rabbit strains following transfer with AdA-I leading to the disappearance of detectable human apo A-I levels within 14 days[61].

18. Development of expression cassettes for hepatocyte-directed gene transfer

The development of potent hepatocyte-specific expression cassettes for gene transfer offers several advantages. First, a therapeutic effect may be obtained at a lower vector dose, thus providing a means to improve the therapeutic index of vectors. Second, hepatocyte-specific expression cassettes represent a very efficient way to restrict transgene expression to hepatocytes, i.e. transcriptional targeting[108, 111, 115]. In contrast, targeting vectors to hepatocytes is significantly more difficult to realise. Third, hepatocyte-specific expression cassettes may lead to immunological unresponsiveness to an immunogenic transgene product via immunological ignorance or immunological tolerance[110, 114, 115, 118, 119].

Optimalisation of transgene expression may be achieved by modulating all levels of expression including transcription, post-transcriptional modification of RNA, RNA export,

RNA stability, and translation. Expression cassettes for hepatocyte-directed transfer have been improved by using new promoter-enhancer combinations[109, 111, 119-123], inclusion of introns[109, 124-127], and inclusion of additional transcriptional sequences like scaffold matrix attachment regions (SMAR) and hepatic control regions (HCR)[126, 128-132]. In a series of studies[87, 108-111, 115, 133], we have performed a direct *in vivo* comparison of nearly 50 different expression cassettes in the context of gene transfer with E1-deleted or E1E3E4-deleted adenoviral vectors using human apo A-I plasma levels in C57BL/6 mice as end-point. These studies represent a continuous improvement of expression cassettes. The conclusion of these investigations is that the *DC172* promoter, consisting of an 890 bp human α_1*-antitrypsin* promoter and two copies of the 160 bp α_1*-microglobulin* enhancer, upstream of the genomic human *apo A-I* sequence and 2 copies of the *HCR-1*, represents at present the most potent expression cassette. After gene transfer with a moderate dose (5×10^{10} particles) of an E1E3E4-deleted vector containing this expression cassette, human apo A-I levels in C57BL/6 mice were more than 3-fold higher than physiological plasma levels in humans[87]. In addition, hydrodynamic gene transfer of minicircles containing this expression cassette resulted in sustained plasma levels in C57BL/6 mice that were equivalent to physiological levels in humans[87].

19. General conclusion

The limited external validity of experimental gene transfer studies with regard to several of the parameters discussed in this review provides a framework to understand why clinical translation of hepatocyte-directed gene transfer is such a major challenge. It is inherent to gene transfer technologies that a wide variety of biological processes affect the ultimate outcome of these interventions. This number of biological determinants is significantly higher compared to classical pharmaceutical therapies or to protein infusion therapies.

Species or strain variation of any of these multiple determinants of the outcome of hepatocyte-directed gene transfer hinders the process of clinical translation. Considering the relative small size of fenestrae in humans, one important parameter that should be considered is the diameter of gene transfer vectors. A progressive increase of fundamental insights into species variations of determinants of the success of hepatocyte-directed gene transfer may provide a solid base for technological advances that may result in more robust technologies and finally in clinical translation. At the present time, it is our view that hepatocyte-directed adeno-associated viral gene transfer has the greatest potential for clinical translation. This view is based on two fundamental properties of these vectors: (1) their size is small enough to pass through human fenestrae that are characterised by a much smaller diameter than those of rodents and (2) innate immune responses are very weak after adeno-associated viral gene transfer. Taken together, the current review contains multiple elements that are a foundation to make stable progress in this field and that provide a realistic perspective on the future of hepatocyte-directed gene transfer.

20. References

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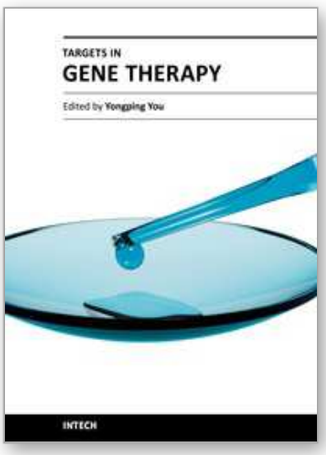

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

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