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ORIGINAL ARTICLE

The size of endothelial fenestrae in human liver sinusoids: implications for hepatocyte-directed gene transfer

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Fenestrae allow the passage of gene transfer vectors from the sinusoidal lumen to the surface of hepatocytes. We have previously shown that the diameter of fenestrae correlates with species and strain differences of transgene expression following intravenous adenoviral transfer. In the current study, we demonstrate that the diameter of fenestrae in humans without liver pathology is 107 ± 1.5 nm. This is similar to the previously reported diameter in New Zealand White (NZW) rabbits (103 ± 1.3 nm) and is significantly smaller than in C57BL/6 mice (141 ± 5.4 nm) and Sprague–Dawley rats (161 ± 2.7 nm). We show that the diameter of fenestrae in one male NZW rabbit and its offspring characterized by a more than 50-fold increase of transgene expression after adenoviral gene transfer is significantly (113 ± 1.5 nm; P < 0.001) larger than in control NZW rabbits. In vitro *filtration experiments using polycarbonate filters with increasing pore sizes demonstrate that a relatively small increment of the diameter of pores potently enhances passage of adenoviral vectors, consistent with* in vivo *data. In conclusion, the small diameter of fenestrae in humans is likely to be a major obstacle for hepatocyte transduction by adenoviral vectors.*

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Keywords: *hepatocyte-directed gene transfer; adenoviral vectors; fenestrae; human liver*

Introduction

Gene transfer to hepatocytes has been extensively pursued to enable secretion of therapeutic gene products into the systemic circulation. Liver sinusoids are highly specialized capillaries within the liver. The sinusoidal endothelium contains pores called fenestrae[1,2] and no basal lamina. Fenestrae are clustered in sieve plates and may provide direct access for circulating gene transfer vectors to the space of Disse, in which microvilli of parenchymal liver cells protrude.[1,3] Whereas fenestrae allow transendothelial passage of gene transfer vectors, endocytosis of vectors by liver sinusoidal endothelial cells[4] and Kupffer cells[4–7] limits hepatocyte transduction.

Previously, we have demonstrated that the size of sinusoidal fenestrae correlates with differences of transgene expression after adenoviral transfer in three different strains of rabbits.[8] The diameter of sinusoidal fenestrae also correlates with interspecies differences of hepatocyte transduction in mice, rats and rabbits after adenoviral transfer.[9] These correlative data support the hypothesis that a small size of fenestrae may restrict transendothelial passage of adenoviral vectors. However, strain and species comparisons are susceptible to genetic confounding factors. Interventions that increase the diameter of fenestrae result in higher transgene expression in New Zealand White (NZW) rabbits,[8,9] providing further evidence for a potential role of the diameter of fenestrae in hepatocyte transduction. Determination of the diameter of fenestrae in humans and comparison with rabbits, mice and rats may provide strategic information for the development of hepatocyte-directed gene transfer in humans.

The exact diameter of fenestrae that is sufficient for transendothelial passage of adenoviral vectors is difficult to predict. Using Vitrobot technology and cryo-electron microscopy, we have previously shown that the diameter of human adenoviral serotype 5 vectors is 93 nm with protruding fibers of 30 nm. Therefore, passage of adenoviral vectors through fenestrae may be influenced by flexibility of the fibers.

Sinusoidal fenestrae may also play a role in hepatocyte transduction during hydrodynamic delivery of DNA.[10] Furthermore, a study on the mechanism of naked DNA clearance after intravenous injection suggested that an increase of the diameter of fenestrae may result in reduced uptake of naked DNA by the liver endothelial cells and enhanced uptake by hepatocytes.[11] Knowledge of the diameter of fenestrae is also relevant for hepatocyte-directed transfer of targeted liposomes that may have diameters from 50 up to 1000 nm.[12]

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The main objective of this study was to determine the diameter of fenestrae in humans with a healthy liver. The second objective of this study was to provide further *in vivo* evidence for modulation of hepatocyte transduction by the size of fenestrae. In this study, we limited the risk of genetic confounding by analyzing the diameter of fenestrae in a substrain of NZW rabbits with significantly increased transgene expression. The third aim of this study was to determine more precisely the diameter of fenestrae that is sufficient for passage of adenoviral vectors. Therefore, passage through pores of filters with reported pore sizes of 80, 100 and 200 nm was quantified. The results of these studies show that the diameter of fenestrae in humans is similar to NZW rabbits. We further show a significantly higher diameter of fenestrae in a substrain of NZW rabbits with increased transgene expression after adenoviral transfer. Finally, results of *in vitro* filtration experiments suggest that adenoviral fibers are flexible.

Results

The diameter of sinusoidal fenestrae in humans is similar to NZW rabbits and significantly smaller than in C57BL/6 mice and Sprague–Dawley rats

Liver biopsies were obtained in six patients undergoing elective laparoscopic cholecystectomy for cholecystolithiasis with no clinical, biochemical or imaging evidence of liver pathology. Four patients were female and two were male. All patients except one Asian woman were Caucasians. Average body mass index was 26 ± 1.2. The average age was 57 ± 6.4 years.

Figure 1a illustrates the frequency distribution histogram of the diameter of sinusoidal fenestrae in humans. Diameters were determined using transmission electron micrographs of plastic liver sections, which cut the sinusoidal wall tangentially. The average diameter was 107 ± 1.5 nm (range 103–113 nm: 103, 104, 105, 107, 109 and 113 nm). Figure 1b shows that this diameter is similar to the previously reported diameter in NZW rabbits (103 ± 1.3 nm)[9] and is significantly smaller than in C57BL/6 mice (141 ± 5.4 nm)[9] ($P < 0.001$) or Sprague–Dawley rats (161 ± 2.7 nm)[13] ($P < 0.001$). Thus, if the correlation between the diameter of fenestrae and transgene expression following adenoviral transfer[8,9] reflects a causal mechanism, these data would imply very low transduction of hepatocytes by adenoviral vectors in humans.

The diameter of sinusoidal fenestrae in a NZW substrain with high transgene expression is significantly larger than in control NZW rabbits

Previously, we have reported very low human apo A-I expression after gene transfer with 4×10^{12} particles per kg of AdA-I in NZW rabbits. AdA-I is an E1E3E4-deleted adenoviral vector containing a hepatocyte-specific expression cassette.[14,15] In the course of these studies, we observed one male NZW rabbit that showed a peak expression at day 10 (110 mg per 100 ml) that was 88-fold higher than in control NZW rabbits (1.2 ± 1.0 mg per 100 ml; $n = 16$) (Figure 2a). The drop of expression levels after day 10 is due to a humoral immune response against human apo A-I (data not shown). This rabbit was used for breeding and generated an F1 offspring with a moderate elevation of peak expression at day 10 after transfer (7.3 ± 1.3 mg per 100 ml; $n = 6$) compared to control New Zealand NZW rabbits (Figure 2b). Crossing the founder rabbit with F1 rabbits generated offspring with high peak expression at day 10 (85 ± 20 mg per 100 ml; $n = 5$) (Figure 2b). If the size of fenestrae plays a causal role in hepatocyte transduction, then the diameter of fenestrae in the founder rabbit and the offspring with high transgene expression is expected to be significantly larger than in control NZW rabbits. Figure 3 illustrates that the frequency distribution histogram of the diameter of fenestrae in this NZW substrain is clearly shifted to the right compared to control NZW rabbits. The average diameter of fenestrae in this substrain (113 ± 1.5 nm; $n = 6$) was significantly ($P < 0.001$) larger than in control NZW rabbits (103 ± 1.3 nm; $n = 10$),[9] consistent with the *a priori* hypothesis. To exclude that we selected rabbits with enhanced expression of the *coxsackie-adenovirus receptor* (CAR) and *α_v-integrin*, real-time reverse transcriptase-PCR was performed. CAR and α_v-integrin mRNA levels normalized to the *glyceraldehyde-3-phosphate dehydrogenase* housekeeping gene mRNA level in the substrain (0.18 ± 0.016 for CAR, 0.029 ± 0.0037 for α_v-integrin; $n = 6$) were not significantly different compared to NZW controls (0.20 ± 0.022 for CAR, 0.032 ± 0.0044 for α_v-integrin; $n = 6$).

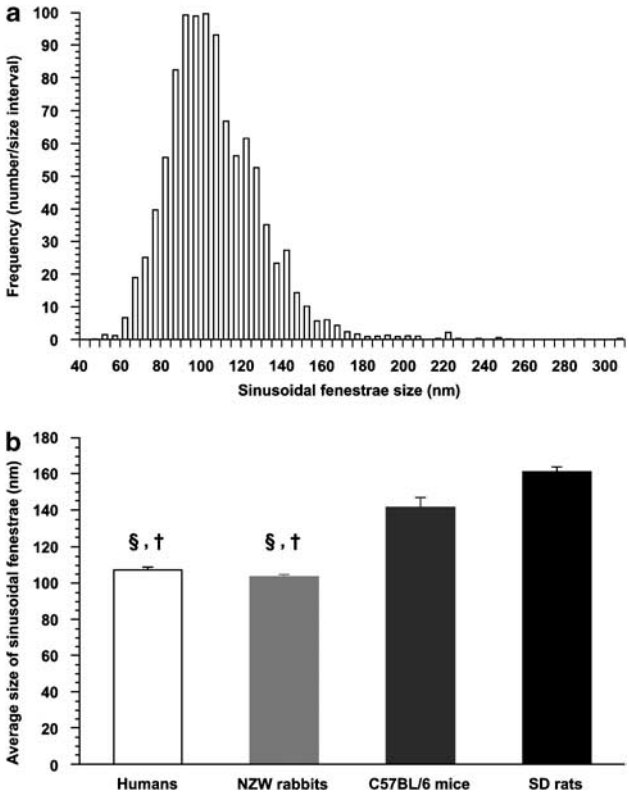

Figure 1 (**a**) Frequency distribution histogram of the diameter of sinusoidal fenestrae in humans with healthy liver ($n = 6$). Each bar corresponds to a 5 nm interval. (**b**) Comparison of the diameter of fenestrae in humans, New Zealand White rabbits, C57BL/6 mice and Sprague–Dawley rats. Data are expressed as means ± s.e.m. $^{\S}P < 0.001$ versus C57BL/6 mice; $^{\dagger}P < 0.001$ versus Sprague–Dawley rats.

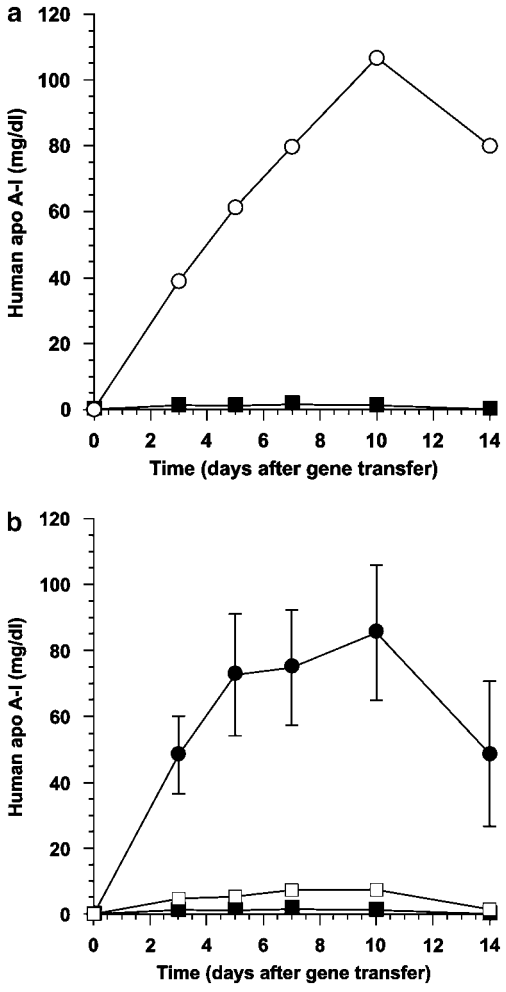

Figure 2 (**a**) Human apo A-I expression after adenoviral gene transfer with 4×10^{12} particles per kg of AdA-I in New Zealand White control rabbits (■; $n = 16$) and in one extreme outlier New Zealand White rabbit with high transgene expression (○; $n = 1$). (**b**) Comparison of human apo A-I expression after adenoviral gene transfer with 4×10^{12} particles per kg of AdA-I in New Zealand White control rabbits (■; $n = 16$), in the F1 offspring (□; $n = 6$) of the extreme outlier and in the offspring of female F1 rabbits and the same founder rabbit (●; $n = 5$). Data are expressed as means ± s.e.m.

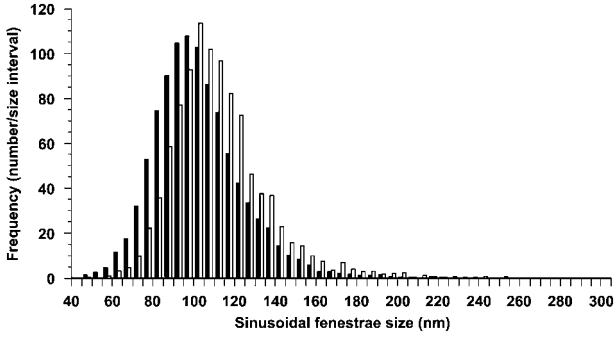

Figure 3 Comparison of the frequency distribution histograms of the size of sinusoidal fenestrae in New Zealand White control rabbits (black bars; $n = 10$) and in substrain rabbits with high transgene expression (white bars; $n = 6$). Each bar corresponds to a 5 nm interval.

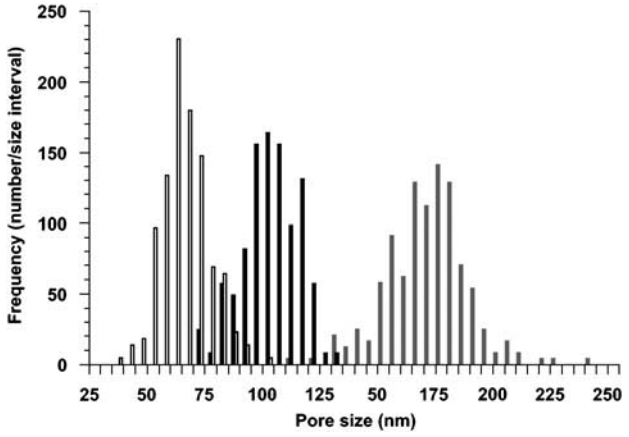

Figure 4 Frequency distribution histograms of the pore sizes of polycarbonate filters with reported pore sizes of 80 nm (white bars), 100 nm (black bars) and 200 nm (grey bars). Each bar corresponds to a 5 nm interval.

Determination of the diameter of pores that is sufficient for the passage of adenoviral vectors in in vitro *filtration experiments*

The data of the current substrain study and of previous intervention studies suggest that a relatively small increase of the diameter of fenestrae underlies a large rise of transgene expression after adenoviral transfer. To obtain more precise information on the threshold of the diameter of pores that allows passage of adenoviral vectors, *in vitro* filtration experiments were performed using polycarbonate filters with increasing pore sizes. The actual average pore size determined by scanning electron microscopy was 66 ± 0.70, 104 ± 1.1 and 172 ± 1.1 nm, respectively, for filters with reported pore sizes of 80, 100 and 200 nm. In contrast with liver tissue, these membranes are supposed not to shrink during preparation for scanning electron microscopy. The frequency distribution histogram of the diameter of the pores of each of these filters, illustrated in Figure 4, shows important interindividual variability for pore sizes of each filter. Figure 5a shows that the recovery of viral particles in the effluent was $0.43 \pm 0.091\%$ ($n = 3$), $51 \pm 7.3\%$ ($n = 3$) and $91 \pm 1.9\%$ ($n = 3$) for polycarbonate membranes with reported pore sizes of 80, 100 and 200 nm, respectively. Scanning electron microscopy of the filter surfaces after filtration showed massive retention for the 80 nm filter (Figure 5b), significant retention for the 100 nm filter (Figure 5c) and virtually no retention for the 200 nm filter (Figure 5d). Taking into account the real diameters of pores in the 100 nm filter, these data are consistent with *in vivo* observations in NZW rabbits showing that a modest increase of pore size can result in a large increase of passage of adenoviral vectors.

Discussion

The main findings of this study are (1) the diameter of fenestrae in humans without liver pathology is similar to that in control NZW rabbits, suggesting that the sinusoidal endothelium is a significant barrier for hepatocyte transduction by adenoviral vectors in

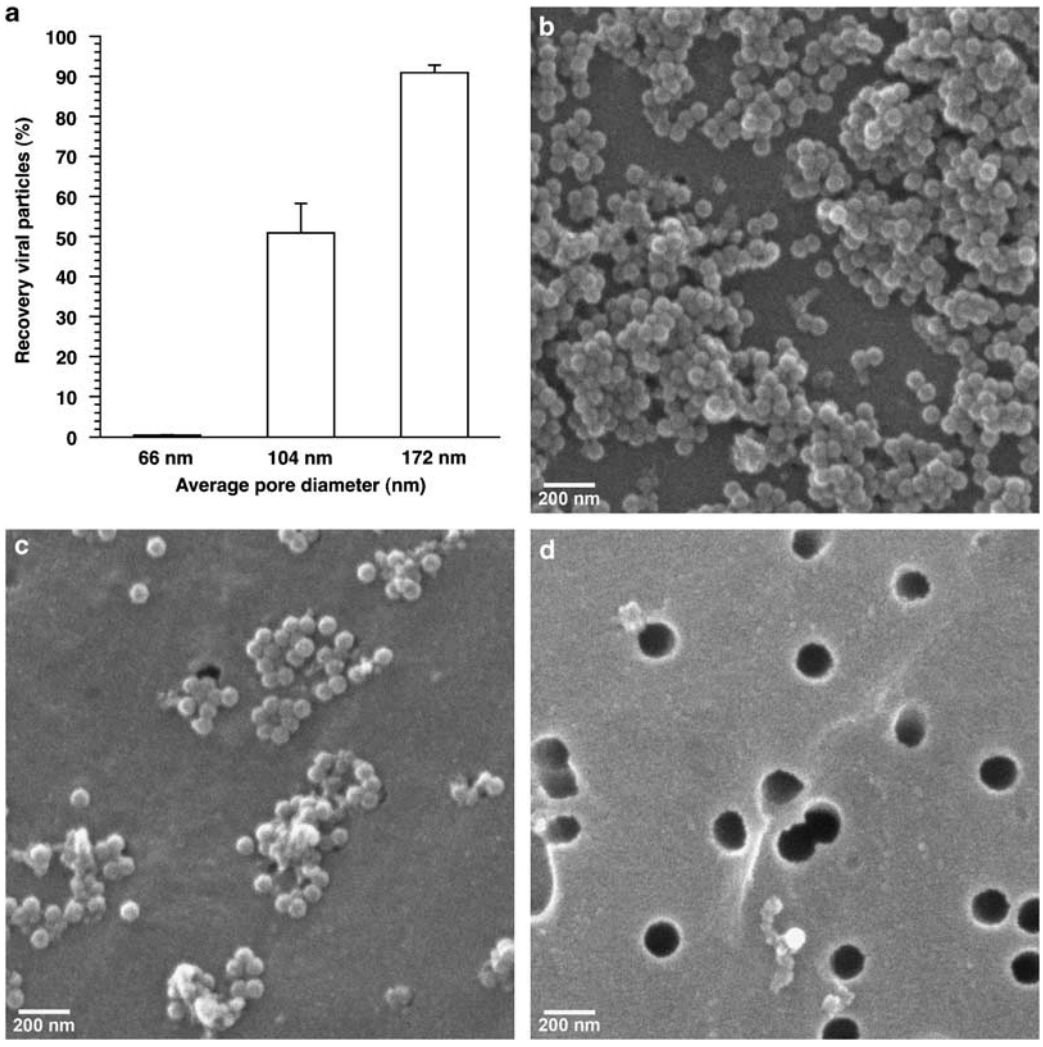

Figure 5 (**a**) Recovery of adenoviral particles in the effluent after filtration of 2×10^{10} adenoviral particles in a volume of 2 ml through polycarbonate filters with different pore sizes ($n = 3$ for each filter type). Representative scanning electron micrographs of filters with reported pore sizes of 80 nm (**b**), 100 nm (**c**) and 200 nm (**d**) following filtration with adenoviral particles.

humans; (2) the size of fenestrae in a substrain of NZW rabbits with high transgene expression is significantly larger than in control NZW rabbits, increasing the likelihood of the hypothesis that the diameter of fenestrae is a critical factor in hepatocyte transduction and (3) *in vitro* filtration experiments are consistent with *in vivo* data showing that a relatively small increase of the diameter of pores may underlie a significant increase of passage of vectors.

Very low transgene expression following adenoviral transfer has previously been observed in control NZW rabbits.[8,9] On the basis of converging evidence that small fenestrae constitute an anatomical barrier for hepatocyte transduction, poor gene transfer into hepatocytes after adenoviral transfer is expected in humans. In the ornithine transcarbamylase deficiency trial, low levels of gene transfer in hepatocytes were detected.[16] The authors concluded that the level of transgene expression was lower than what would have been predicted on the basis of preclinical models.[16] Histological alterations of the livers in patients with partial ornithine transcarbamylase deficiency may have contributed to low hepatocyte transduction. Nevertheless, 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. Innate immune responses to adenoviral capsids[16,17] have seriously undermined the potential of adenoviral vectors for hepatocyte-directed transfer. Although strategies to mitigate or abrogate these innate immune responses have been designed[18–20] the small size of fenestrae in humans may constitute a more significant obstacle for hepatocyte-directed transfer with adenoviral vectors. We have previously shown that an increase of the diameter of fenestrae induced by intraportal injection of sodium decanoate[8] or by intravenous injection of N-acetylcysteine combined with transient liver ischemia[9] potently enhances transgene expression in NZW rabbits. However, these strategies are too complex and not sufficiently safe for clinical applications. A convenient drug to increase the diameter of fenestrae is currently not available. The small size of fenestrae in humans must also be taken into account for the development of non-viral hepatocyte-directed transfer using naked DNA or liposomes.[10–12] In addition, the range of average diameters of fenestrae in humans observed in this study

(103–113 nm) may be broad enough to result in significant interindividual differences of hepatocyte transduction.

Accurate measurements of sinusoidal fenestrae can only be performed using transmission electron microscopy of plastic-embedded specimens, as specimen preparation for scanning electron microscopy induces substantial (approximately 30%) shrinkage effects.[21] Sample processing, image acquisition and image analysis were consistently performed according to standardized operating protocols until the final result was obtained. Therefore, determinations of the diameter of fenestrae between different species and different strains in this study are unbiased and comparisons of diameters are reliable.

Liver biopsies in this study were obtained from patients undergoing laparoscopic cholecystectomy with a body mass index of less than 30, without clinical, biochemical or imaging evidence of liver abnormalities and without known malignancy. Liver fibrosis and cirrhosis,[22] alcoholic liver disease without cirrhosis[23] and aging[24] may lead to a reduced permeability of the sinusoidal endothelium by reduced number of fenestrae and the development of a basal lamina. As the objective of this study was to evaluate the diameter of fenestrae in patients with normal liver, we also excluded patients with a history of malignant tumors. In patients with colorectal cancer without evidence of liver metastases at the time of tumor resection, hepatic perfusion abnormalities that are predictive for the subsequent occurrence of liver metastases have been described.[25–27] Although it is unsure whether these perfusion abnormalities affect the diameter of fenestrae, patients with malignancy were excluded for this reason.

Previously, we have demonstrated that the differences of the diameter of sinusoidal fenestrae correlate with differences of hepatocyte transduction in different strains of rabbits[8] and in different species.[9] Strain and species comparisons are susceptible to genetic confounding factors. In this study, we show a significantly larger diameter of fenestrae in one male NZW rabbit and its offspring with very high transgene expression compared to control NZW rabbits. In contrast, no differences in CAR and α_v-integrin mRNA levels in the liver were observed. As CAR protein expression on the cell surface correlates positively with CAR mRNA expression[28] and α_v-integrin mRNA levels correlate with $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins at the protein level,[29] these results indicate that significant differences in primary and secondary adenovirus receptor levels between the substrain rabbits and controls are unlikely. The observations in the NZW substrain therefore significantly increase the likelihood of the hypothesis that the diameter of fenestrae is a critical factor in hepatocyte transduction. However, we cannot exclude that the breeding process selected other unknown phenotypic characteristics that may have contributed to enhanced hepatocyte transduction.

The *in vitro* filtration experiments are consistent with *in vivo* data showing that a relatively small increase of the diameter of fenestrae may lead to a large increase of hepatocyte transduction. The interpretation of the data obtained from the filter with a reported pore size of 100 nm should take into account that the actual average diameter of these pores is 104 nm. Second, perfusion through these filters was performed under high pressure. Significantly, less vectors will pass in the *in vivo* situation under low sinusoidal pressure. Nevertheless, the recovery of 51% of adenoviral vectors in the effluent after filtration through polycarbonate membranes with a reported pore size of 100 nm indicates significant flexibility of the fibers. Indeed, adenoviral serotype 5 fibers are characterized by two flexibility domains.[30]

In conclusion, the rabbit data in this study provide further support for the hypothesis that the diameter of fenestrae is a dominant determinant of hepatocyte transduction by adenoviral vectors. The development of viral and non-viral hepatocyte-directed gene transfer in preclinical models in mice and rats should take into account that the diameter of fenestrae in humans is significantly smaller, limiting the external validity of data obtained in these rodents.

Materials and methods

Generation and propagation of AdA-I

The construction, propagation and purification of the E1E3E4-deleted adenoviral vector *AdA-I* has been described previously.[14,15,31] *AdA-I* contains a 1.5 kb human α_1-*antitrypsin* promoter upstream of the 2 kb genomic human *apo A-I* sequence and four copies of the 154 bp human *apo E* enhancer.

Animal experiments

All experimental procedures in animals were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee. NZW rabbits were obtained from the University of Gent (Merelbeke, Belgium) and breeding colonies were maintained at the animal facility of the University of Leuven. Gene transfer experiments (4×10^{12} v.p. kg^{-1}) through the marginal ear vein were performed at the age of 12 weeks.

Human apo A-I ELISA

Human apo A-I levels were determined by sandwich ELISA as described previously.[32]

Human liver biopsies

Liver biopsies ($n = 6$) were obtained from patients without signs of primary and secondary liver disease undergoing elective laparascopic cholecystectomy. Exclusion criteria were a body mass index above 30 and the presence of any malignancy. A unit of 1 cm^3 biopsies were cut from the distal rim of the left lobe and immediately placed in phosphate buffered saline supplemented with 0.05% heparine at 37 °C before fixation for electron microscopy analysis. All patients gave informed consent. The study was approved by the Medical Ethical Committee of the University Hospital Gasthuisberg Leuven.

Filtration and quantification of adenoviral particles in vitro

Viral particles were diluted in 0.9% NaCl to obtain a final concentration of 1×10^{10} particles per ml. Subsequently, a volume of 2 ml viral suspension was applied on polycarbonate membranes with reported pores of 80, 100 or 200 nm (Poretics Corporation, Livermore, CA, USA). These filters have a thickness of 5–12 μm and the

pores are cylindrical. A fixed gravitational force of 4.94 N was applied. This force generates the minimal pressure required to perfuse virus-free saline through a polycarbonate filter with pores of 80 nm. Following perfusion, effluents were recovered for quantification of viral particles and filter membranes were used for scanning electron microscopy analysis.

Adenoviral DNA was isolated from the effluent using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. In parallel, DNA extractions were performed for negative controls and standards. Baxter water and virus-free saline were used as negative controls. Standards were prepared by serial dilution of E1E3E4-deleted adenoviral vectors in saline. Particle concentrations in the standards ranged from 1×10^3 to 1×10^{10} particles per ml. A glyceraldehyde-3-phosphate dehydrogenase plasmid[33] was used to spike samples, negative controls and standards as an internal control.[34]

The number of viral particles per ml effluent was determined by real-time quantitative PCR (qPCR core kit, Eurogentec, Seraing, Belgium) using the adenovirus fiber forward primer 5′-gcgcaagaccgtctgaagat-3′, the adenovirus fiber reverse primer 5′-aggcacagttggag gaccg-3′ and the adenovirus fiber probe 5′-caaccccgtg tatccatatgacacggaaat-3′. The coefficient of variation of the glyceraldehyde-3-phosphate dehydrogenase copy number determined by real-time quantitative PCR was less than 1%, indicating that variation in DNA extraction efficiency was negligible.

Quantification of CAR and α_v-integrin expression

CAR and α_v-integrin mRNA levels in the liver were quantified by real time reverse transcriptase-PCR as described previously.[8]

Quantification of the size of sinusoidal fenestrae in the liver by transmission electron microscopy

Perfusion of rabbit livers for electron microscopy analysis was performed as described previously.[8,9] Human biopsies were perfused manually using a modified version of this protocol. In brief, 1 cm^3 biopsies from the distal rim of the left lobe were collected in phosphate buffered saline supplemented with 0.05% heparin at 37 °C immediately after excision. Subsequently, a fluid filled 18-G needle was inserted alongside the outer rim of the biopsy, retracted 1 mm and fixation was performed by injecting 1.5% glutaraldehyde fixative buffered in 0.067 M cacodylate at pH 7.4 and 37 °C into the tissue. Perfusion was halted after the color had changed from dark reddish brown to yellow brown and the consistency from soft to stiff. Injections were repeated from different angles until successful perfusion was obtained. A unit of 1 mm thick slices were cut into 30–40 1 mm^3 blocks, washed in 0.067 M cacodylate buffer pH 7.4 and transferred to a 1% OsO_4 fixative solution buffered with phosphate buffered saline 0.1 M pH 7.4 for subsequent immersion fixation during 1 h at 4 °C. After washing in phosphate buffered saline 0.1 M pH 7.4, dehydration was carried out rapidly in graded ethanol series (70–100%), followed by embedding in Epon. Subsequent steps were identical to processing of rabbit samples.[9] Specimens were examined in a Philips CM 100 microscope (FEI, Eindhoven, The Netherlands) at 80 KV. The size of fenestrae was measured as the largest diameter in sections that cut the endothelial wall tangentially and show the fenestrae as complete holes. Measurements were performed manually on a monitor using ImageJ software (Wayne Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). The average number of measurements per biopsy was 822 ± 90 in rabbits and 528 ± 142 in humans. The technique of manual fixation of wedge biopsies was validated in NZW rabbits. The diameter of fenestrae in rabbits was similar following manual fixation of wedge biopsies compared to perfusion fixation of the whole liver.

Scanning electron microscopy of polycarbonate membranes

Polycarbonate filters were cut in two pieces to visualize both sides of the filter surface and were subsequently treated as described previously.[35] Scanning electron micrographs were obtained at University of Maastricht (EM unit, Pathology) using a Philips XL30 microscope (FEI, Eindhoven, The Netherlands) at an accelerating voltage of 30 KV.

Statistical analysis

Data are expressed as mean ± s.e.m. Comparison of peak human apo A-I levels and the diameter of sinusoidal fenestrae in rabbits was performed by Student's *t*-test using Instat V3.0a (Graph Pad Software, San Diego, CA, USA). Interspecies comparison of the diameter of fenestrae was performed by analysis of variance followed by Tukey's multiple comparisons test (Graph Pad Software). A two-sided *P*-value of less than 0.05 was considered statistically significant.

Acknowledgements

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