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RESEARCH ARTICLE

Factors influencing immune response after *in vivo* retrovirus-mediated gene transfer to the liver

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Abstract

Background Highly efficient retrovirus-mediated gene transfer into hepatocytes *in vivo* triggers an immune response directed against transduced hepatocytes. This effect may be due either to spreading of retroviral vectors in the blood stream with subsequent infection of antigen presenting cells (APCs) or to cross-presentation of the transgene product present as a contaminant in the viral stock. In order to decrease immune response, we evaluated the effect of asanguineous perfusion of the liver as well as purification of the viral stock on long-term transduction of hepatocytes using the nls-lacZ marker gene.

Methods Animals were divided in four groups. In group 1, the viral supernatant was perfused in the regenerating liver after complete vascular exclusion of the organ. In group 2, using the same strategy, animals received retroviral supernatant that was passed through a β-galactosidase affinity column to reduce β-galactosidase contamination. In two control groups (respectively groups 3 and 4) the corresponding viral supernatants were delivered via peripheral injection.

Results In group 1, 23.1% of animals had no immune response 2 months after gene delivery vs. 33.4% in group 2, 4.3% in control group 3, and 0% in control group 4. Statistical analysis of the results demonstrated that only the difference between groups 2 and 3 was statistically significant. This indicated that both asanguineous perfusion together with passage through an affinity column were required to decrease significantly immune response.

Conclusions Our present results suggest that both supernatant contamination and viral spreading contribute to immune response after retrovirus-mediated gene delivery to the liver. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords liver; gene therapy; retroviral vector; immune response; cross-presentation

Introduction

Gene therapy for liver inherited diseases should ideally result in indefinite synthesis of a therapeutic protein. Many strategies have been devised during the past years to attain this goal, but were faced with two major difficulties. The first one was to reach a high level of transgene expression in order to reverse a disease phenotype. The second one was to achieve long-term expression of the transgene without induction of an immune response directed against the transgene product. We have focused our interest on *in vivo* gene transfer to the liver using recombinant retroviral vectors. These retroviral vectors

are delivered to the portal blood stream during liver regeneration induced by two-thirds partial hepatectomy [1]. Using this strategy, we previously showed that the level of transduction correlated to the extent of liver regeneration as well as to the viral titer [2,3]. However, it turned out that using high-titer retroviral vectors, the level of liver transduction was high but resulted in short-term expression of the transgene [4,5]. We recently reported that this transient expression was due to the generation of a cytotoxic immune response directed against the transgene product that resulted in elimination of transduced hepatocytes [6].

Although the mechanism whereby retroviral vectors trigger the immune response is still not completely understood, it has been reported that this type of vector could infect antigen presenting cells (APCs) after intramuscular delivery [7,8]. However, other mechanisms such as cross-presentation of the therapeutic protein to the immune system may also occur. Indeed, such cross-presentation was described for adeno-associated virus (AAV)-based vectors which do not infect directly APCs [9,10]. Therefore, the presence of the transgene-encoded protein in the viral preparations may be responsible for induction of immune response via cross-presentation [11].

In the present study we evaluated two strategies aimed at reducing immune response following retrovirus-mediated liver gene delivery using the *E. coli* β-galactosidase protein coupled to a nuclear localisation signal as a reporter. We first reasoned that complete vascular exclusion of the liver at the time of virus delivery could reduce spreading of viral vectors throughout the blood stream and hence could decrease infection of cells outside the liver including extra-hepatic APCs. Secondly, to decrease cross-presentation of the transgene product, viral supernatant was passed through an affinity column to eliminate β-galactosidase contamination. We show that the combination of the two strategies was required to achieve sustained β-galactosidase expression in the absence of immune response in a statistically significant number of animals.

Materials and methods

Animals and surgical procedures

Male rats from the congenic Wistar Furth strain and weighing 180–200 g were purchased from Iffa Credo (L'asbresle, France). Animals were maintained under a 12-h light cycle and fed *ad libitum*. All surgical procedures were conducted on deeply anesthetised animals according to the guidelines of the French Ministère de l'Agriculture. Rats were anesthetised with isofluran inhalation (3% v/v in air).

Two-thirds partial hepatectomy was performed by removing the two main liver lobes after ligation at the hilum according to the procedure of Higgins and Anderson [12]. Retroviral delivery was performed 24 h after partial hepatectomy. Asanguineous perfusion of the regenerating liver after complete vascular exclusion was carried out as previously described [1]. Rats were laparotomised and the liver was excluded from the systemic blood stream by clamping the portal vein and the supra-hepatic and infra-hepatic vena cava. The gastroduodenal vein was ligated and sectioned. The portal vein was cannulated and 20 ml of viral supernatant were infused over 5 min. The viral supernatant was harvested from the infra-hepatic vena cava. The liver was rinsed with 6 ml of saline and the blood circulation was restored after suturing the vessels. A blood sample was drawn minutes after declamping and the virus titer of the serum was assayed by end-point dilution using Te671 cells and counting the number of positive colonies after X-Gal staining as described [1]. Peripheral delivery was carried out by direct injection via a peripheral vein of 2 ml of retrovirus-containing medium.

Seven days after retroviral injection, liver biopsies were performed. Rats were anesthetised and laparotomised to expose the remnant liver lobes. A liver fragment (approx. 10 mg) was obtained from one lobe. Each liver biopsy sample was paraffin-embedded after fixation in formalin.

Retroviral vectors

The TELCeB6 AF7 cell line (referred to as TA7) produced recombinant retroviral vectors carrying a nls-lacZ reporter gene encoding *E. coli* β-galactosidase coupled to a nuclear localisation signal (nls) [13]. The reporter gene transcription was under the control of the retroviral long terminal repeat (LTR).

A 24 h recombinant retroviral supernatant was harvested from the confluent producer cell line and filtered through a 0.45-µm membrane. Before use, 8 µg/ml of polybrene (hexame-thedrine bromide (Sigma, St. Louis, MO, USA) was added to the supernatant.

Titers were determined by end-point dilution using Te671 cells and the number of blue colonies after X-Gal staining were counted. The titer was routinely 5×10^7 colony-forming unit (cfu)/ml.

Affinity column

In order to reduce β-galactosidase present in the viral supernatant, the retroviral supernatant was passed through a HiTrap affinity column (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) coupled with a rabbit anti-β-galactosidase polyclonal antibody (Chemicon, Temecula, CA, USA) using a peristaltic pump with a flow rate of 0.4 ml/min. After each use the column was regenerated by rinsing with 1 ml of sodium acetate buffer (0.1 M, pH 4). The same column was used throughout the study.

The β-galactosidase activity was determined by enzymatic fluorimetric assay using 4 MUG (methylumbelliferyl β-D-galactoside) as a fluorescent substrate. Supernatant was incubated with 0.1mg/ml of 4 MUD (25 mM Tris

HCl, pH 7.5, 125 mM NaCl, 2 mM $MgCl_2$, 12 mM 2-mercaptoethanol, 4 MUG, water) for 30 min at 37 °C. Reaction was stopped by 50 ml freezed 25% trichloracetic acid and the fluorescence was read at 460 µm.

Immunohistochemistry

The presence of β-galactosidase in hepatocytes was assessed by immunohistochemistry on formalin-fixed/paraffin-embedded sections (5 µm). Sections were deparaffinised and endogenous peroxidase activity was inhibited by incubation in a 3% H_2O_2 solution in phosphate-buffered saline (PBS). Polyclonal primary anti-β-galactosidase antibody diluted 1 : 2000 in PBS was applied overnight at 4 °C. Positive cells were visualised with biotinylated anti-mouse immunoglobulin and streptavidin/peroxidase using AEC as a chromogenic substrate. Slides were counterstained with hematoxylin and the percentage of positive cells was calculated on different fields at ×40 magnification.

Antibody detection

The presence of antibodies directed against β-galactosidase was carried out in rat serum by an ELISA assay. In brief, 96-well dishes were coated overnight at 4 °C with commercially available β-galactosidase (Sigma) at 10 mg/ml. After rinsing with PBS/Tween (0.5%, v/v) serial dilutions of the serum were incubated for 1.5 h at 37 °C. After washing with PBS/Tween, the presence of antibodies was revealed using biotinylated anti-rat IgG immunoglobulin and streptavidin/peroxidase.

Statistical methodology

Mann-Whitney's test was used to compare quantitative data between groups. Fischer's exact test was used for categorical data. An analysis of covariance was used to compare the percentage of purification, adjusted on the rank of pass through the column.

Results

A first group of animals (group 1, $n = 13$) was subjected to partial hepatectomy according to the procedure devised by Higgins and Anderson [12] and received the next day retroviral vectors containing the nls lacZ gene [13] via asanguineous perfusion of the regenerating liver. Perfusion of the liver after complete vascular exclusion was carried out as previously described [1]. The liver was excluded from the systemic blood flow by clamping the portal vein, the hepatic artery and the supra-hepatic and infra-hepatic vena cava. The portal vein was cannulated and 10 ml of viral supernatant (infectious titer: 5×10^7 particles/ml) were infused over 5 min. During the perfusion, the viral supernatant was harvested from the infra-hepatic vena cava. The liver was rinsed with 6 ml of saline and the blood circulation was restored after suturing the vessels. A second group of animals (group 2, $n = 18$) received via asanguineous perfusion the same amount of retroviral supernatant that had been passed through an affinity column coupled with a rabbit anti-β-galactosidase polyclonal antibody to remove contaminating β-galactosidase. Control animals received via bolus injection in a peripheral vein 2 ml of either basic viral vector preparation (group 3, $n = 22$) or viral supernatant passed through the affinity column (group 4, $n = 3$). In each group, the transduction of hepatocytes was assessed randomly in some animals in a small surgical biopsy harvested by laparotomy performed at day 7. In these biopsies, the number of β-galactosidase-positive hepatocytes was evaluated by immunohistochemistry. As shown in Table 1, the mean number of positive hepatocytes in the biopsies was variable between groups. However, the differences between groups were not statistically significant (ANOVA, $p = 0.1$). We also observed that the positive hepatocytes were less evenly distributed after asanguineous perfusion than after direct injection in the liver lobes. From day 28 after gene delivery, the presence of anti-β-galactosidase antibodies was assessed by ELISA in the serum as previously described [6]. Animals that did not mount an antibody response after 60 days were considered tolerant (Table 1). In these animals, the 1 : 50 serum dilution was negative for the presence of antibodies by ELISA whereas in all non-tolerant animals the 1 : 5000 serum dilutions were strongly positive. The presence of such antibodies is a *bona fide* indicator of anti-β-galactosidase immune response, and in our previous studies the humoral immune response was always associated with cytotoxic elimination of transduced cells [6]. This may be due to the absence of β-galactosidase secretion since, in other situations, such

Table 1. Summarised data of the animal groups

Group	Administration protocol	n	% Positive hepatocytes day 7	% Tolerant rats*	% Positive hepatocytes day 60 in tolerant animals*
1	Perfusion without column	13	5.8 ± 7.1	23.1	8.8 ± 3.2
2	Perfusion with column	18	12 ± 7.1	33.3	7.5 ± 8
3	Injection without column	22	5.8 ± 2.3	4.5	n.d.
4	Injection without column	3	1.7 ± 1	0	n.d

n = number of experimental animals.
*Tolerant rats are rats without antibodies (negative result at 1 : 50 serum dilution) and with β-gal-positive hepatocytes at day 60.
n.d. = not determined.

as factor IX gene delivery using AAV vectors, antibody formation against the transgene product was not always associated with a cytotoxic response [10]. Here again, in animals that had anti-β-galactosidase antibodies, no β-gal-positive hepatocytes were detected at the time of sacrifice indicating that a cytotoxic immune response had taken place. In contrast, in tolerant animals, β-gal-positive hepatocytes were still present at sacrifice (day 60) with no significant quantitative differences with the values observed at day 7 (Table 1). The results shown in Table 1 also showed that the overall number of tolerant animals was higher after asanguineous perfusion of the liver (group 1, 23.1% tolerant) as compared with direct injection (group 3, 4.5% tolerant). However, careful statistical analysis revealed that the difference between groups 1 and 3 was not significant (Fischer's exact test, $p = 0.134$). Similarly, the difference between group 2 (asanguinous perfusion with affinity column) and the control group 4 was not significant (Fischer's exact test, $p = 0.526$). Conversely, the number of tolerant rats between group 2 (asanguineous perfusion and passage through the affinity column) and group 3 (33.3 vs. 4.5%) was statistically significant (Fischer's exact test, $p = 0.033$) indicating that both vascular exclusion and β-galactosidase elimination were necessary to decrease immune response significantly. Finally, passage through the affinity column alone was not sufficient to impair immune response since the difference between the two control groups 3 and 4 (direct injection without or with affinity column) was not significant (Fischer's exact test, $p = 1$).

To better understand the impact of each parameter, we first analysed the effect of vascular exclusion on the spreading of viral vectors. To this end, in 29 animals that underwent asanguineous liver perfusion and in 5 control rats injected via a peripheral vein, we harvested a serum sample 5 min after declamping (asanguineous perfusion) or after injection (direct injection) and the number of viral particles in the serum was determined by titration on Te671 cells (assuming a total blood volume of 10 ml for the rats). The values were compared with the total number of viral particles delivered to the animals. As shown in Table 2, a significant difference was observed between the two administration modes and vascular exclusion resulted in a higher decrease in systemic viral particles (250-fold) than direct injection (20-fold; $p = 0.014$, Mann-Whitney's test). This indicated that asanguineous perfusion actually reduced the spreading of vectors outside the liver. Nevertheless, after vector administration, viremia were quite similar between injected (5×10^5 infectious particles/ml of serum) and perfused animals (2×10^5 infectious particles/ml). This may be due to the fact that a higher input was used for asanguineous perfusion (10 ml of viral vectors, i.e. 5×10^8 infectious particles) compared with injected rats (2 ml of viral vectors, i.e. 10^8 infectious particles).

We next analysed the effect of eliminating β-galactosidase protein from the viral supernatant by passing it through an affinity column. β-Galactosidase activity was determined in each viral supernatant before and after passage through the affinity column. The viral titer did not change after passage through the column. The purification rate was calculated as the ratio between the enzyme activity before passage over the activity after passage through the column, and the results from group 2 animals are shown in Table 3. Analysis of the results revealed a correlation between the extent of β-galactosidase purification rate and the absence of immune response (Table 3, Figure 1; Mann and Whitney test, $p = 0.032$). However, this difference was no more significant when the rank of passage through the column was not taken into account. In other words, there was a significant effect of the rank of passage through the column ($p = 0.03$) and the number of tolerant rats was higher after injection of the first supernatants passed through the column. This effect was significant for all passages through lower than 8 and not after (threshold effect) indicating that the potency of the column to eliminate β-galactosidase was reduced after the eight

Table 2. Influence of delivery technique on viral dissemination

Mode of administration	Viral input (infectious particles)	Circulating particles[a] (infectious particles)	Fold decrease
Peripheral injection	10^8	5×10^6	20*
Asanguineous perfusion	5×10^8	2×10^6	250

[a]Circulating particles were evaluated in blood samples drawn minutes after injection or after declamping for asanguineous perfusion. The circulating particles were calculated by multiplying the viral titer obtained by the blood volume of the animal (10 ml).
*Values are significantly different, $p = 0.014$ using the Mann and Whitney test.

Table 3. β-Galactosidase purification rate and immune response in animals after asanguinous perfusion (group 2)

Rank of passage	Purification rate (fold decrease)	Presence of immune response in recipient animal (at day 60)[a]
1	70	no
2	100	no
3	100	yes
4	–	no
5	65	no
6	120	no
7	81	yes
8	32	yes
9	60	yes
10	35	yes
11	48	yes
12	35	yes
13	64	yes
14	64	yes
15	66	yes
16	50	no
17	50	yes
18	41	yes

[a]The presence of an immune response corresponds to the existence of circulating antibodies together with the absence of transduced hepatocytes in the liver at sacrifice (60 days after gene transfer)

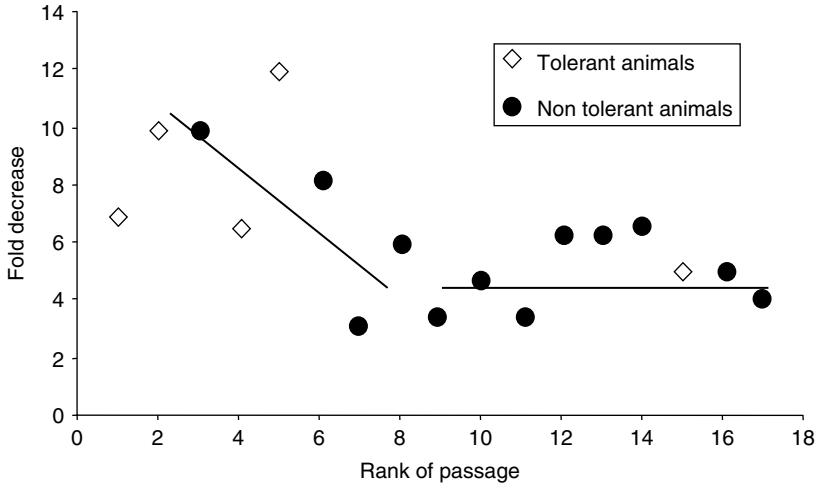

Figure 1. Correlation between the purification rate (fold decrease) and the rank of passage through the column. The rank pass effect was assessed using a comparison of the variance before and after the eighth rank pass through the column. An analysis of covariance was used to compare the percentage of purification, adjusted on the rank of passage through the column. A piecewise linear regression showed a threshold effect after the eighth passage in the column

passage in spite of the procedure used to regenerate the column after each passage. These results were confirmed by the analysis of the homosedasticity. The variance was significantly different before and after the eighth rank of pass through the column (ratio variance test $F_{(7,8)ddl} = 5.23$; $p = 0.0329$) indicating a threshold effect of the rank of pass. Therefore, a piecewise linear regression was conducted before and after the eighth rank of pass (Figure 1). This variability was not found after the eighth rank pass, demonstrating a differential evolution over rank of pass.

Discussion

In the present report we show that combination of vascular exclusion of the liver at the time of virus delivery together with elimination of β-galactosidase from the vector preparation lead to decreased immune response and hence to sustained expression of the transgene in the liver. These results support the view that multiple mechanisms are responsible for induction of an immune response against the transgene product after virus-mediated gene delivery. Our data clearly demonstrate a statistical correlation between purification of the viral stock by affinity column and decreased immune response in injected animals. Since the production of retroviral vectors usually involves the harvest of cell culture supernatant, the viral preparations are likely to contain large amounts of the transgene-encoded protein if an ubiquitous promoter is used. In that case, expression of the transgene in the packaging cells results in the presence of the corresponding protein in the cell supernatant which may be cross-presented after being injected. In contrast, when using a tissue-specific promoter, one can avoid expression of the transgene in the viral preparation and hence decrease cross-presentation after *in vivo* delivery of the viruses. The purification of the viruses by using an affinity column resulted in a dramatic decrease of β-galactosidase contamination. This purification was necessary but not sufficient alone to decrease the immune response, demonstrating that cross-presentation participated in the induction of the immune response, at least to some extent.

Similarly, reduction of virus spreading in the blood flow by using asanguineous liver perfusion was not able to significantly impact immune response, although there was a tendency toward a decreased immune response (23.1% tolerant animals in group 1 vs. 4.3% tolerant animals in group 3). The level of circulating viral particles was higher than we expected after vascular exclusion and extensive wash of the liver before declamping. It seemed likely to us that viral particles loosely attached to hepatocytes during perfusion were released into the systemic blood stream after unclamping even though the liver was rinsed after virus perfusion. Since the circulating level of viral particles is only 2.5-fold lower after asanguineous perfusion than after direct injection, this may explain why the difference in the proportion of tolerant animals between group 1 (23.1%) and group 3 (4.3%) was not statistically significant. It seems likely that a higher diminution in virus spread should become effective and we hypothesise that such specific delivery to the liver using asanguineous perfusion may help to prevent infection of extra-hepatic APCs by decreasing the number of infectious circulating particles. Although this is not formally demonstrated here, it has been shown previously that retrovirus vectors can infect APCs to trigger an immune response [14]. Furthermore, after vascular delivery, it has been demonstrated that retroviruses could infect cells in the spleen and bone marrow [15]. Therefore, combination of vascular exclusion together with supernatant purification could result in decreased APC infection and lower β-galactosidase cross-presentation with ensuing lower induction of immune response.

Such induction of immune response via two different mechanisms is reminiscent of the situation with other vectors such as AAVs. Indeed, previous studies reported that cross-presentation was primarily responsible for the induction of immune response after AAV-mediated gene delivery to the muscle [10,16]. However, it was subsequently shown that infection of APCs such as immature dendritic cells by AAV vector with a ubiquitous promoter could activate cytotoxic T lymphocytes and eliminate transduced muscle cells [17–19]. The site of injection as well as the dose administered could impact on antigen-specific immune response [20,21]. Finally, the use of a tissue-specific promoter could decrease immune response against the transgene product after AAV gene delivery [17,22]. We believe that the same holds true for retroviral vectors and that cross-presentation of the transgene product as well as APC infection are key components of the immune response.

It is now becoming increasingly clear that techniques aimed at avoiding immune response are mandatory for the design of successful *in vivo* gene therapy protocols. Along this line, we now show that improving vector purification and allowing proper delivery to the target organ are helpful to achieve long-term expression in retrovirally transduced liver. The recent demonstration of a complete and stable correction of a genetic disease via retrovirus-mediated gene transfer to the liver in a genetic model resulting from a single point mutation and where no immune response is expected [23] highlights the immune response as an ultimate hurdle before applications of liver gene transfer are successful.

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