Recombinant adenovirus administration in rat peritoneum: endothelial expression and safety concerns

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Abstract

Background. Initial studies of adenovirus-mediated gene transfer to the peritoneum have shown transgene expression in the mesothelium from the parietal peritoneum. Using a replication-deficient adenovirus encoding β-galactosidase (AdβGal), we investigated the expression efficiency and the distribution of the transgene to different areas of both visceral and parietal peritoneum and to extra-peritoneal tissues.

Methods. Male Wistar rats received an intraperitoneal injection of 15 ml of 0.9% NaCl alone or containing 1 × 10⁹ or 3 × 10⁹ p.f.u. of AdβGal. Evaluations of the histology of the peritoneum, the transgene expression and the safety of adenovirus-mediated gene transfer, using measurement of both βGal activity and staining, were performed 1, 3 and 5 days post-injection.

Results. At 1 day post-injection of 3 × 10⁹ p.f.u. of AdβGal, significant βGal activity and staining were detected in the omentum and mesenteric peritoneum. βGal staining was observed in endothelial cells, mesothelial cells and adipocytes. Focal mononuclear infiltrates restricted to the submesothelial area of the visceral peritoneum were also observed. No expression was detected in the mesocolon and parietal peritoneum, where the mesothelium was damaged. Significant βGal activity and staining were observed in lymph nodes, lungs, liver, heart and kidneys, in the absence of inflammatory changes.

Conclusions. Intraperitoneal delivery of adenoviral vectors allows highly efficient transgene expression in mesothelial cells, but also in endothelial cells and adipocytes of the visceral peritoneum. Adverse focal mononuclear infiltrates, as well as spreading of the adenoviral vector from the abdominal cavity to the systemic circulation, were observed in parallel. Transgene expression in endothelial cells is potentially important since the latter play a key role in the alterations of the peritoneal membrane associated with long-term peritoneal dialysis. However, these data emphasize the need for less immunogenic adenoviral vectors, ideally containing an endothelial cell-specific promoter, to overcome immune response-related problems and spreading to extra-peritoneal tissues.

Keywords: adenovirus; endothelium; gene transfer; mesothelium; peritoneum

Introduction

Gene transfer in the peritoneal cavity holds considerable promise for the investigation of mediators or pathways operating in the peritoneal membrane (PM) and, perhaps, participation in the management of a variety of conditions such as peritoneal dialysis (PD), adhesions or intra-abdominal malignancies [1]. In the past few years, in vivo gene transfer to the peritoneal cavity using adenoviral vectors has been used in rat and mouse models. Intraperitoneal (i.p.) administration of such vectors resulted in transgene expression restricted to the peritoneal mesothelium [2–4], and in the transduction of multiple tissues in the developing murine fetus [5].

The endothelium lining peritoneal capillaries represents the functional barrier during PD [6] and an obvious target for gene transfer in the PM. On the other hand, the PM is characterized by a large, highly vascularized surface area (~1 m²) and an abundant lymphatic drainage [7]. These characteristics raise the feasibility of endothelial targeting and the issue of safety and dissemination of adenoviral vectors administered i.p. Using two separate methods, we investigated in detail the expression and distribution of the transgene to different areas of both visceral and peritoneal peritoneum as well as to extra-peritoneal tissues.
Materials and methods

Recombinant adenovirus

Adenovirus encoding β-galactosidase (AdβGal) was kindly provided by Dr R. Gerard. Culture and purification of recombinant adenovirus were performed as previously described [8].

In vivo gene delivery in rat peritoneum

Male Wistar rats (400 g) were obtained from Harlan CBP (Zeist, The Netherlands). All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Ethics Committee for animal studies. The rats received a single 15 ml i.p. injection of either 0.9% NaCl (control group; n = 6) or 1 × 10^9 (n = 6) or 3 × 10^9 p.f.u. (n = 7) of AdβGal diluted in 0.9% NaCl. One day before the i.p. injection, and daily until sacrifice, the animals received an intramuscular injection of 4mg/kg dexamethasone (Aacidexam, NV Organon, Oss, The Netherlands). Animals were put under light anaesthesia with sevoflurane (Abbott NV, Ottignies, Oss, The Netherlands). Animals were put under light anaesthesia with sevoflurane (Abbott NV, Ottignies, Belgium) when the i.p. injection was administrated. No mortality resulting from that protocol was recorded. Rats were sacrificed at day 1 or 5 post-i.p. injection.

Histological evaluation of peritoneum

Samples from the omentum, mesenteric and mesocolon regions of the visceral peritoneum and from the anterior and posterior abdominal cavity of the parietal peritoneum were obtained 1, 3 and 5 days post-injection, then processed for paraflin embedding and sectioned. The integrity of the mesothelium was carefully assessed by observation of ciliated cells or immunostaining using a monoclonal anti-Pan cytokeratin (Sigma, St Louis, MO).

Evaluation of transgene expression

At 1, 3 and 5 days post-injection, samples from the different regions of the visceral and parietal peritoneum were removed and fixed for 2 h in a solution of 4% formaldehyde in phosphate-buffered saline (PBS) pH 7.4, washed in PBS pH 7.4 before being incubated at 37°C for 24 h in a solution of 5mM K_4Fe(CN)_6, 5 mM K_3Fe(CN)_6, 2 mM MgCl2 and 0.025% of Igepal in PBS pH 7.4 containing 500 μg/ml of X-Gal substrate, and finally rinsed in PBS pH 7.4. After macroscopic analysis, peritoneum samples were post-fixed, embedded in paraflin, sectioned, and counter-stained with haematoxylin–eosin. Cells positive for βGal staining displayed a blue nuclear/perinuclear staining. To assess the percentage of positive transduced cells, the total and stained cells were counted in six separate fields of each section.

Safety of in vivo adenovirus-mediated gene transfer in peritoneum

Homogenates of rat tissue samples from the duodenum, stomach, small intestine, colon, pancreas, liver, lung, spleen, kidney and heart were centrifuged at 1000 g for 10 min at 4°C, then the supernatants were centrifuged further at 17000 g for 20 min at 4°C. The final supernatants were kept for βGal assay (Invitrogen, San Diego, CA). βGal activity was expressed in βGal units per mg of protein.

The concentrations of total proteins, GOT, GPT, lipase, amylase and urea from blood samples were determined by standard automated analysis [9].

Results

Morphological evaluation of peritoneum

Detailed histological examination of the parietal (anterior, posterior left and right abdominal wall) and visceral (omentum, mesenteric and mesocolon regions) peritoneum was performed at 1, 3 and 5 days post-injection (Figure 1). None of the injected rats and visceral peritoneum showed massive infiltrate and oedema suggestive of acute peritonitis or focal areas of vascular proliferation.

Rats injected with 1 × 10^9 p.f.u. of AdβGal showed a normal morphology and a lack of infiltrate at day 1
Evaluation of transgene expression: determination of βGal activity and staining

Higher βGal activity was observed in animals injected with $3 \times 10^9$ p.f.u. compared with $1 \times 10^9$ p.f.u. of AdβGal. At 1 day post-injection of $3 \times 10^9$ p.f.u. AdβGal, the omentum and mesenteric peritoneum exhibited significant βGal activity, while no significant activity was detected in the mesocolon and parietal peritoneum (Figure 2). In extra-peritoneal tissues, βGal activity was considerably increased in liver, lung and kidney, and slightly increased in heart (Figure 2). At 5 days post-injection of $3 \times 10^9$ p.f.u. AdβGal, the βGal activity in peritoneum samples and other extra-peritoneal tissues was considerably lower (data not shown).

At any day post-injection, the visceral peritoneum of rats injected with $1 \times 10^9$ or $3 \times 10^9$ p.f.u. of AdβGal revealed positive βGal staining at the macroscopic level, with high, moderate and low βGal staining in the omentum, mesenteric region and mesocolon, respectively. At the microscopic level, the typical blue nuclear/perinuclear βGal staining was observed in mesothelial cells and adipocytes within the omentum (Figure 3A), in endothelial cells lining the peritoneal capillaries and venules (Figure 3C), and in the whole visceral peritoneum (Figure 3E). No βGal staining was observed in tissues originating from control rats injected with buffer alone (Figure 3B, D and F).

Semi-quantitative analysis of omentum sections from rats injected with $1 \times 10^9$ or $3 \times 10^9$ p.f.u. of AdβGal revealed positive βGal cells ranging from 70% at 1 day post-injection to 40% at 5 days post-injection. No βGal staining was observed in parietal peritoneum sections.

Extra-peritoneal transgene expression

Lymphatic nodes from the peritoneal cavity of rats injected with AdβGal showed massive βGal staining of the ganglionic cells (>90% of cells), contrasting with the negligible endogenous βGal staining in control rats (Figure 4A and B). A significant βGal staining was also observed in the spleen (strong reaction in the capsule), liver, lungs, kidney and heart (Figure 4C–G). In the lungs, low and dispersed βGal staining was observed in alveolar walls (Figure 4C). In the kidneys, βGal staining was observed mainly in the proximal tubule cells of nephrons located in the outer cortex (Figure 4D). In the liver, βGal staining was intense in the capsule, and included numerous hepatocytes in the hilar region (Figure 4E and F). Isolated cardiomyocytes were also detected in the heart (Figure 4G). No inflammatory infiltrates were observed in the lungs, kidneys, liver and heart.

Fig. 2. βGal activity in PM and tissues. βGal activity measured 1 day post-injection of $3 \times 10^9$ p.f.u. AdβGal in sample tissues from the same rat. The results are expressed as fold stimulation of βGal activity compared with control and are representative of those obtained with two animals. Determinations were done in duplicate.

Fig. 3. Staining for βGal in the visceral peritoneum of control and adenovirus-treated rats. Representative sections of the omentum (A–D) and visceral peritoneum (E and F) 1 day after injection with $3 \times 10^9$ p.f.u. of AdβGal (A, C and E) or buffer alone (B, D and F). Specific βGal staining (indicated by blue nuclei) is observed in the mesothelial cells (A and E), endothelial cells (A and C) and adipocytes from adenovirus-treated rats. The lack of staining indicates the lack of endogenous βGal activity in tissues from control rats. βGal staining in all panels. Original magnification: (A–F) ×300.
Several studies have reported the feasibility of in vivo adenovirus-mediated gene transfer into rat peritoneal cavity [1–4]. Thus far, the transgene expression has been restricted to the mesothelium [2–4]. In view of the vascularization and lymphatic drainage of the PM, the aim of this study was to evaluate the potential to induce transgene expression in the endothelium lining peritoneal capillaries, as well as adverse effects and other safety concerns that might arise from the i.p. administration of adenoviral vectors.

The experimental procedure used to deliver the adenoviral vector to the PM may influence the nature of the cells transduced and the efficiency of the process. Previous studies reported that a single i.p. injection of recombinant adenovirus (1–5 × 10^9 p.f.u.) in a small volume (50 μl to 1 ml) induced transgene expression in mesothelial cells at and around the site of injection and into the muscular abdominal wall [2,3,12]. Our data show that delivery of similar doses of adenovirus in a higher volume (15 ml) ensured a better distribution and a higher transgene expression to a large peritoneal membrane area. Transgene expression was found in endothelial cells, adipocytes and mesothelial cells of the omentum, as well as in the mesenteric and mesocolon regions of the visceral peritoneum. In fact, 70% of cells within the omentum, the most superficial region of the peritoneum, were transfected at day 1 post-injection of AdβGal. The similar transgene expression using two different doses of adenoviruses suggests that the volume of infusate, rather than the dose of adenovirus, has a strong impact on the transgene distribution. The observation that transgene expression in the visceral peritoneum was much higher at day 1 than at day 5 post-injection confirmed the intrinsic property of adenoviral vector to induce transient gene transfer.

Our data show that the transgene expression in the visceral peritoneum involves mesothelial cells, as reported by others [3,12], but also adipocytes and endothelial cells. The endothelium lining peritoneal capillaries represents the major functional barrier to solute exchange during PD [6,7]. In that respect, transgene expression in endothelial cells could offer important prospects, since endothelial cell proliferation, and modifications in essential components such as endothelial nitric oxide synthase or the water channel aquaporin-1, are involved in the pathophysiology of the alterations of the PM associated with long-term PD [13]. In contrast to previous studies [3,11], no transgene expression could be observed in the mesothelial cells lining the parietal peritoneum. The loss of mesothelial cells in this part of the peritoneal membrane is probably due to the protocol of large volume infusion rather than related to tissue dissection and processing (the mesothelial cells are well preserved in the visceral peritoneum).

The use of adenoviruses has been associated with the development of a dose-dependent inflammation at the site of delivery. This acute inflammation can usually be suppressed by dexamethasone administration [14]. The administration of high-dose dexamethasone in our study prevented most of the inflammatory response after injection of AdβGal. Discrete and very limited

Table 1. Serum chemistry in control and adenovirus-injected rats

<table>
<thead>
<tr>
<th></th>
<th>Total proteins (g/dl)</th>
<th>GOT (U/l)</th>
<th>GPT (U/l)</th>
<th>Lipase (U/l)</th>
<th>Amylase (U/l)</th>
<th>Urea (mg/dl)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>6.7 ± 0.2</td>
<td>169 ± 61</td>
<td>57 ± 15</td>
<td>8 ± 3</td>
<td>1770 ± 1218</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>3 × 10^9 p.f.u.</td>
<td>6.5 ± 0.3</td>
<td>172 ± 54</td>
<td>61 ± 21</td>
<td>10 ± 3</td>
<td>1724 ± 1054</td>
<td>42 ± 10</td>
</tr>
</tbody>
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Rats were injected i.p. with either 0.9% NaCl (15 ml) or 3 × 10^9 p.f.u. AdβGal (in 15 ml of 0.9% NaCl) and blood samples were analysed after 1 day. Experimental data are the mean ± SD for four animals in each condition.

Serum chemistry analysis

Serum concentrations of total proteins, GOT, GPT, lipase, amylase and urea from rats injected with 3 × 10^9 p.f.u. of AdβGal were not significantly different from those obtained with control rats at 1 (Table 1), 3 and 5 (data not shown) days post-injection.

Discussion

In vivo gene therapy offers possibilities to improve the understanding or treatment of structural damage induced in the PM [2,3,10,11].
cellular infiltrates were observed in the visceral peritoneum (Figure 1C and D), whereas no infiltrate could be documented in the parietal peritoneum. Previous studies have shown that the cytotoxicity (due to cell arrest in G2/M phase) and the immune response observed after adenovirus delivery could be partially due to the adenovirus and the transgene [15].

The safety of i.p. delivery of adenoviral vectors was assessed using βGal enzymatic activity and staining. We observed increased βGal activity and positive staining in heart, kidney, liver and lung, in the absence of inflammatory infiltrate. These data confirm that liver and lung are the preferential targets for recombinant adenovirus following systemic or i.p. injection [5,12,16]. Since the peritoneum has abundant lymphatic drainage [2,5] and as the intraperitoneal fluid volume has a direct impact on lymphatic clearance [17], delivery in a large volume of fluid could explain the spreading of the vector to extra-peritoneal tissues. This assertion was supported by the analysis of visceral peritoneal ganglia, showing the presence of the transgene in ~90% of the ganglionic cells compared with minimal endogenous βGal activity in control rats. Furthermore, lymphatic drainage was also reported to account for adenoviral spreading in other adenoviral-mediated gene delivery models [18]. Serum chemistry analysis from rats injected with AdβGal was similar to control animals, in agreement with a previous study [16].

Despite spreading of the adenoviral vector to extra-peritoneal tissues, transgene expression in endothelial cells was associated with the use of a large volume of infusate. New strategies will be required to increase transgene expression in peritoneal endothelial cells, for instance by using new less immunogenic adenoviral vectors containing an endothelial cell-specific promoter.

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Conflict of interest statement. None declared.

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