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Adenovirus-Mediated Gene Transfer into Selected Liver Segments Using a Vascular Exclusion Technique

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Key Words

Animal model, gene transfer · Liver · Recombinant adenovirus · Gene transfer · Green fluorescent protein Transgene expression · Liver toxicity

Abstract

Adenovirus-mediated gene therapy is hampered by severe virus-related toxicity, especially to the liver. The aim of the present study was to test the ability of a vascular exclusion technique to achieve transgene expression within selected liver segments, thus minimizing both viral and transgene product toxicity to the liver. An E1-E3-deleted replication-deficient adenovirus expressing a green fluorescent protein (GFP) reporter gene was injected into the portal vein of BDIX rats, with simultaneous clamping of the portal vein tributaries to liver segments II, III, IV, V, and VIII. GFP expression and inflammatory infiltrate were measured in the different segments of the liver and compared with those of the livers of animals receiving the viral vector in the portal vein without clamping. The GFP expression was significantly higher in the selectively perfused segments of the liver as compared with the non-perfused segments ($p < 0.0001$) and with the livers of animals that received the vector in the portal vein without clamping ($p < 0.0001$). Accordingly, the inflammatory infiltrate was more intense in the selectively perfused liver segments as compared with all other groups ($p < 0.0001$). Fluorescence was absent in lungs and kidneys and minimal in spleen. The clinical usefulness of adenovirus-mediated gene transfer to the liver largely depends on the reduction of its liver toxicity. Clamping of selected portal vein branches during injection allows for delivery of genes of interest to targeted liver segments. Transgene expression confined to selected liver segments may be useful in the treatment of focal liver diseases, including metastases.

Introduction

Originally, gene therapy approaches were designed to replace defective or missing genes in a specific cell population [1]. Currently, their spectrum has broadened, and monogenetic diseases, such as haemophilia, adenosine deaminase deficiency, hypercholesterolaemia, and ornithine transcarbamylase deficiency [2, 3], or plurigenetic

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diseases, such as cardiovascular diseases [4], diabetes [5], infectious diseases [6], and especially cancer [7, 8], are potential targets for gene therapy.

Physicochemical and viral vectors are currently used to introduce transgenes into target cells. Physicochemical vectors like plasmids, naked DNA, and liposomes are characterized by a low in vivo efficiency and a limited toxicity. Viral vectors (retrovirus, lentivirus, adeno-associated virus, herpes simplex virus, hepatitis virus, and adenovirus) are commonly used gene delivery systems in preclinical and clinical studies. Each type of virus has its own advantages and disadvantages. The recombinant human adenovirus type 5 is frequently used for in vivo gene therapy applications [9–11]. This virus can infect very efficiently a wide range of dividing and non-dividing cells. The adenovirus-related toxicity, however, remains a major concern. Adenovirus in vivo will generate not only a humoral immune response with production of neutralizing antibodies, but also a predominantly T helper 1 immune response because of expression of adenoviral proteins at the surface of infected cells. Moreover, the protein encoded by the transgene can trigger an immune response that can aggravate the adenovirus-related toxicity [12, 13].

Ideally, transgene expression should be limited to the tumour site and its vicinity in order to increase the treatment efficacy and to reduce the incidence of side effects. Selective delivery of viral vectors should fulfil these two goals.

Different routes of gene delivery to liver tumours have been studied: direct intra-tumoral injection, systemic intravenous delivery, and intraportal or intra-arterial (hepatic artery) injection [10, 11, 14, 15]. Both intraportal and systemic intravenous injection of recombinant adenoviral vectors successfully led to transgene expression in hepatocytes [3, 16]. The therapeutic efficacy of virus-mediated gene transfer to the liver has been shown in animal models of cancer, diabetes, hypercholesterolaemia, adenosine deaminase deficiency, and other diseases [17–19].

In the present study, we evaluated the potential benefits of the use of adenovirus-mediated transfer of the green fluorescent protein (GFP) reporter gene to selected segments of the liver using a portal vein vascular exclusion technique. As compared with portal vein delivery without vascular clamping, the herein described vascular exclusion technique improved transgene expression to the targeted liver segments, at the expense of a more intense inflammation locally.

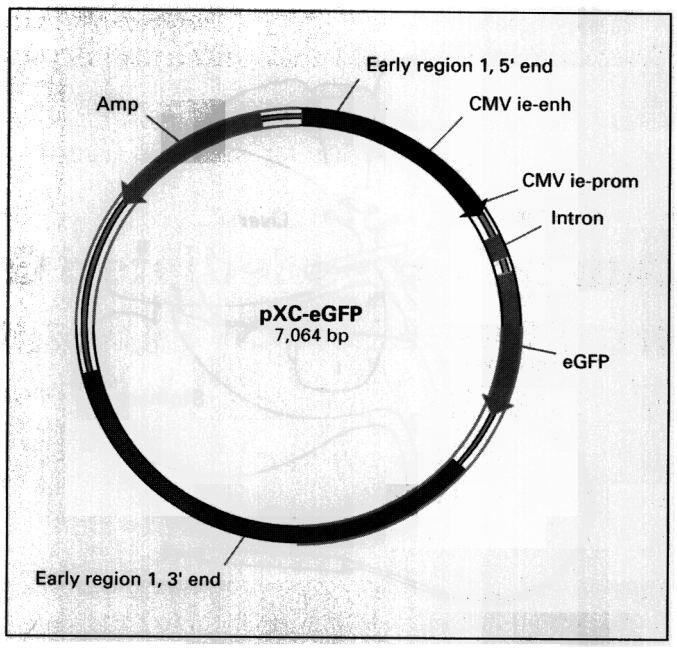


Fig. 1. Adenoviral transfer vector expressing the enhanced green fluorescent protein (eGFP) reporter gene. The expression of the eGFP open reading frame (ORF) is controlled by the immediate-early CMV promoter-enhancer (CMV ie-enh, CMV ie-prom). To optimize expression, the eGFP ORF is placed downstream of an artificial intron (Intron) derived from pCIneo. This eGFP expression cassette is flanked at its 5′ end by the 5′ early region 1 of adenovirus 5 and at its 3′ end by the 3′ end of the early region 1 of adenovirus 5. The E1A and B adenoviral ORF are deleted in this vector derived from pXC15 [21].

Materials and Methods

Cells

293 cells and HR911 cells (IntroGene, Leiden, The Netherlands) were maintained in Dulbecco's minimal essential medium (DMEM; Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Life Technologies) and 1% penicillin-streptomycin (10,000 IU/ml; Life Technologies) at 37 °C in an atmosphere of 5% CO_2.

Adenoviral Vector Construct

Ad/eGFP is an E1-E3-deleted, replication-defective, recombinant human adenovirus type 5 expressing the enhanced GFP (eGFP) under the control of the immediate-early cytomegalovirus (CMV) promoter. To generate this virus, the eGFP-coding region was first excised as an Nhe1-Xho1 DNA fragment from peGFP-C1 (Clontech Laboratories, Basel, Switzerland) and ligated into pCIneo-eGFP – encompassing the CMV promoter, the artificial intron of pCIneo, the coding region of eGFP, and the polyA addition signal of pCIneo – which was then inserted into pXC15–18 digested with BamH1 and Hpa1 to produce the adenoviral transfer vector pXC-eGFP (fig. 1) [20, 21]. Ad/eGFP was then generated by homologous recombination in 293 cells following cotransfection by the calcium phosphate proce-

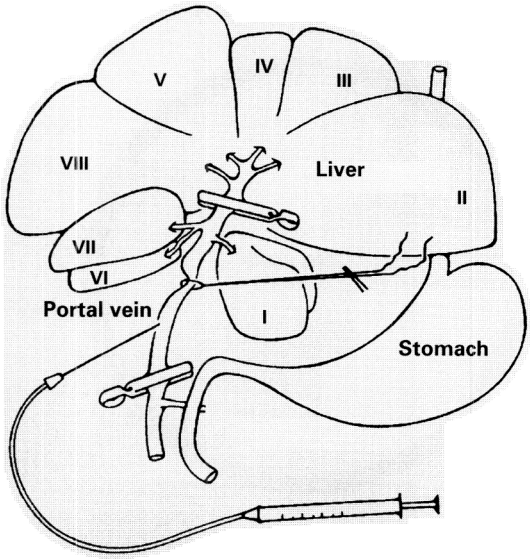


Fig. 2. Anatomy of the rat liver. Clamping of the portal tributaries in order to perfuse selectively segments I, VI, and VII of the liver. A vascular clamp is placed proximal to the injection site to prevent backflow of Ad/eGFP. [From ref. 23, with permission.]

dure using pJM17 and pXC-eGFP. Viruses collected 10 days after transfection were further plaque purified three times on HR911 cells. Viruses from three independent plaques were analyzed by restriction enzyme digestion of viral DNA purified from 293 cells by the Hirt procedure [22]. One of them was selected and used to produce large stocks on 293 cells. Viruses were purified by two rounds of CsCl centrifugation. After the second centrifugation, the virus band was collected and dialyzed at 4°C against three changes (at least 200 vol each) of 10 m*M* Hepes, pH 8.0, and 150 m*M* NaCl in a Slide-A-Lyzer (0.5–3.0 ml capacity) gamma-irradiated 10 K dialysis cassette (Pierce, Rockford, Ill., USA).

Plaque assays were performed in six-well dishes with 30% confluent HR911 cells. The cells were infected with 400 µl of virus suspension in DMEM containing 10% FCS and antibiotics for 1 h. The inoculum was then replaced by 2.5 ml of freshly prepared overlay, equilibrated at 40°C. The overlay contained 1 vol of 2 × MEM (a fivefold dilution of 10 × MEM; Life Technologies), 20% FCS, antibiotics, and 1 vol of 2.5% seaplaque agarose (FMC BioProducts, Rockland, Me., USA). After the agarose solidified, the dishes were returned to the CO_2 incubator. Plaques were scored 5 days after infection.

Animal Model and Surgical Procedure

All experiments were performed in accordance with the animal guidelines at the University of Lausanne Medical Center, Switzerland.

Male 8- to 10-week-old BD IX rats weighing 250–280 g (Iffa-Credo, l'Arbresle, France) were anaesthetized with a single intraperitoneal injection of pentobarbital (45 mg/kg).

The liver of the rat is divided in eight segments (fig. 2) [23]. Segments I–III are on the left side with a clear separation between segments I and II. Segment IV, the median segment, separates the left-sided segments from the right-sided segments. Located on the right side are segments V–VIII. Segments V and VIII are clearly separated from the inferior segments VI and VII. The vascular structures (hepatic artery and portal vein) and the bile duct run in the hepatoduodenal ligament and divide to supply each individual segment. The separation between the different segments and their vessels allows for an easy vascular access to specific segments.

On day 0, a midline laparotomy was performed, and the portal vein and its branches were exposed. The animals were divided into four groups. In group 1 (n = 6), a vascular clamp (Bulldog Clamp, Harvard Apparatus, South Natick, Mass., USA) was placed across the portal tributaries to the upper liver segments (II–V and VIII). Ad/eGFP (10^{10} particle-forming units) was injected in 0.1 ml phosphate-buffered saline (PBS; Gibco-BRL/Life Technologies, Inchinnan, UK) into the portal vein using a 29-gauge needle. Backflow of the virus solution in the splenic and mesenteric veins was prevented by placing a clamp on the portal vein proximal to the site of injection. After needle removal, haemostasis was obtained through mechanical compression and application of tissue glue (Histoacryl®; Aesculap, Tuttlingen, Germany). The proximal vascular clamp was removed, and the distal clamp between the upper and the lower segments of the liver was removed 3 min later.

In group 2 (n = 4), the same operative procedure was applied, but no clamp was placed distally on the portal vein in order to perfuse all liver segments with Ad/eGFP.

In two control groups, 0.1 ml PBS was injected into the portal vein with (group 3; n = 3) and without (group 4; n = 3) simultaneous clamping of the portal tributaries to the upper liver segments.

GFP Transgene Expression in Different Visceral Organs

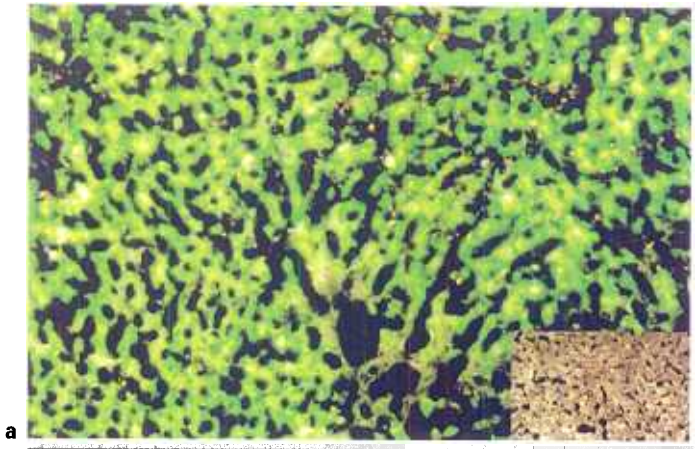
The rats were sacrificed 5 days after virus delivery. They were perfused intravenously with 100 ml PBS and 100 ml 4% paraformaldehyde. Liver, lungs, kidneys, and spleen were harvested and fixed in 4% paraformaldehyde at 4°C. Forty-eight hours before tissue section, tissue samples were placed in 25% sucrose and postfixed in Cryomatrix® (frozen embedding resin; Shandon, Pittsburgh, Pa., USA). Cryosections of 7 µm were performed. Assessment of cells expressing GFP was made by counting fluorescent cells under a microscope equipped with a green fluorescent filter (Axioplan 2; Zeiss, Feldbach, Switzerland) using three different fields at a high magnification (× 200) [24]. For each organ, three different sections were analyzed.

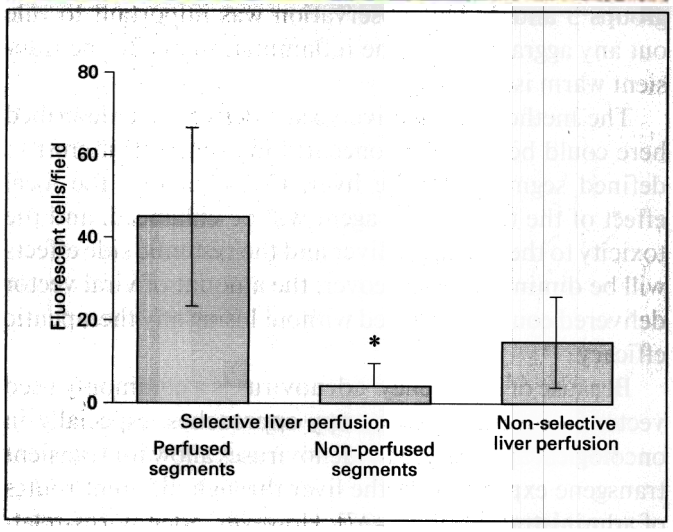
Liver Inflammation after Intravenous Ad/eGFP Delivery

After paraformaldehyde fixation and paraffin embedding, 5-µm sections of liver, lungs, kidneys, and spleen were stained with haematoxylin and eosin. Mononuclear cells were counted on three fields at × 100 magnification of three different sections for every segment of the liver.

Statistics

Statistical analyses were performed by use of a linear mixed model, and p values were adjusted by the Turkey-Kramer correction (SAS procedure 'mixed', version 6.12; SAS Institute, Cary, N.C., USA). Data are expressed as mean values ± SD.

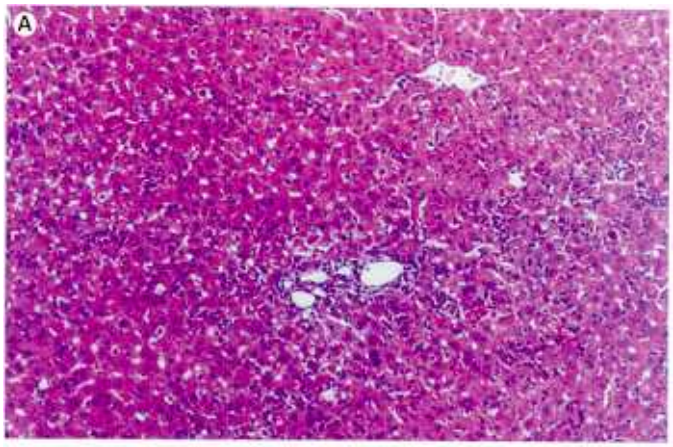


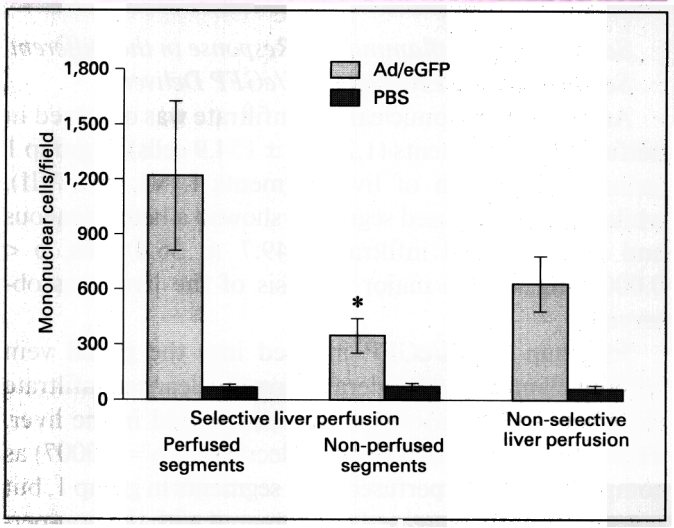


a

b

Fig. 3. a Fluorescence in a selectively perfused segment of the liver with simultaneous clamping of the portal tributaries (group 1) and negative control. Up to 80% of the liver parenchyma is fluorescent. Cryosection, green fluorescent filter. × 75. **b** Fluorescent cells per counted field in perfused and non-perfused segments of the liver (selective perfusion) and in perfused segments without clamping (whole-liver perfusion; * $p < 0.0001$). Transgene expression (GFP) is mainly present in the targeted liver segments.





a

b

Fig. 4. a Representative mononuclear cell infiltration in a perfused (part A) and in a non-perfused (part B) liver segment with simultaneous vascular clamping during intra-portal injection of Ad/eGFP. An intense cell infiltrate is visible in the perfused segment (part A), while only marginal and inhomogenous cell infiltrate was detected in the non-perfused segment (part B). HE. × 75. **b** Mononuclear cell infiltrates after selective and non-selective perfusion with Ad/eGFP or PBS (* $p < 0.0001$). The main inflammatory response is localized in the targeted segments.

Results

GFP Transgene Expression in the Different Segments of the Liver

Fluorescence was measured in 3 animals from group 1 (selective perfusion of the liver with Ad/eGFP), in 3 animals from group 2 (intraportal injection of Ad/eGFP), and in 3 animals (PBS injection in portal vein) from both control groups.

The results of transgene expression are shown in figure 3. In group 1, 45.9 ± 7.2 and 10.5 ± 5.7 fluorescent cells were counted in the perfused segments (I, VI, and VII) and in the occcluded segments (II–V and VIII), respectively. The difference was statistically significant ($p < 0.0001$; fig. 3b). Up to 80% of the cells in the perfused segments expressed the GFP transgene (fig. 3a).

In group 2, fluorescent cells were heterogeneously distributed in every segment. An average of 21.5 ± 7.8 fluorescent cells was counted. This difference was statistically significant as compared with segments that were selectively perfused in group 1 ($p < 0.001$; fig. 3b).

No fluorescent cells were detected in the lungs and the kidneys, while few fluorescence-positive cells (0.9 ± 0.4) were observed in the spleens in both groups 1 and 2. Animals from groups 3 and 4 (PBS injection) did not show any fluorescence in any organ.

Severity of the Inflammatory Response in the Different Segments of the Liver after Ad/eGFP Delivery

An intense mononuclear cell infiltrate was observed in perfused liver segments (1,223.3 ± 154.9 cells) in group 1 (selective perfusion of liver-segments I, VI, and VII), while the non-perfused segments showed a heterogeneous and moderate cell infiltrate (349.7 ± 36.4 cells, $p < 0.0001$; fig. 4). No major necrosis of the liver was observed.

In group 2 (Ad/eGFP injected into the portal vein without clamping), a moderate mononuclear cell infiltrate (653.5 ± 73.9 cells) was evenly distributed in the liver. This infiltrate was significantly decreased ($p = 0.0007$) as compared with the perfused liver segments in group 1, but not significantly increased as compared with the non-perfused segments in group 1 ($p = 0.17$).

No pathological cell infiltrate was present in the livers harvested from the control groups. In group 3 (PBS injection into the portal vein and simultaneous vascular clamping), 72 ± 14 cells were counted per field in the perfused segments and 74 ± 16 cells in the non-perfused liver segments (NS). The animals from group 4 (PBS injection in the portal vein) showed 59.8 ± 22 cells per field. The two control groups were not statistically different (NS). The number of mononuclear cells in the livers of the two control groups (3 and 4) was significantly lower as compared with groups 1 and 2 ($p < 0.0001$).

Discussion

In the present study, we have achieved higher transduction rates in targeted segments of the liver by intraportal injection of an adenovirus bearing a fluorescent reporter gene and selective clamping of portal tributaries. The transduction efficiency was significantly higher in the targeted segments of the liver when the method of selective delivery was used as compared with intraportal administration of the virus without clamping. The treatment efficacy is straightly dependent on the level of transgene expression. Increased reporter gene expression measured in the perfused liver segments is directly related to the clamping of the portal vein tributaries; this accordingly decreases the diffusion volume. Corresponding with that, the inflammation observed in these segments was more robust, but no major necrosis was seen. The transient peri-operative vascular clamping of the portal tributaries during 3 min did not increase the inflammatory response in the upper liver segments, as shown in the two control groups 3 and 4. This observation was important to rule out any aggravation of the inflammation due to the transient warm ischaemia.

The method of selective vector delivery we described here could be used for concentrating genes of interest to defined segments of the liver. Consequently, the local effect of the therapeutic agent will be enhanced, and the toxicity to the rest of the liver and the systemic side effects will be diminished. Moreover, the amount of viral vector delivered could be reduced without losing any therapeutic efficacy.

Because of its potency, adenovirus is a commonly used vector in several gene therapy approaches, especially in oncological applications. Adenoviruses allow for transient transgene expression in the liver through different routes of administration [9, 25–27]. However, adenovirus-related toxicity caused by expression of viral proteins and transgene products remains a major concern. Administration of adenovirus will induce a humoral immune response with production of neutralizing antibodies that will prevent the efficacy of further systemic virus delivery. Moreover, adenovirus-induced hepatitis and systemic inflammatory response syndrome can be tremendous and even lethal [10, 28]. Current research on adenovirus

mainly focuses on ways to reduce this toxicity. *Gutless* adenoviruses in which the entire viral genome is deleted are being developed. The absence of genes encoding for viral proteins has been shown to dramatically reduce the immunogenicity of this vector [29]. Construction of genetically modified targeted viral particles and the use of tissue-specific promoters that allow for transgene expression in targeted cells only are other fields of intense endeavour [30, 31]. Systemic injection of adenovirus leads to high non-specific transduction in the liver at the expense of a high toxicity [15]. Direct intratumoral injection of the viruses is mainly used for transfer of immunomodulatory genes and allows for local gene expression [11]. Finally, the surgical procedure associated with the mode of vector administration can target organs or part of organs [14].

The herein described surgical approach for gene transfer into selected segments of the liver using a vascular exclusion technique is currently tested in animal models of metastatic cancer. In humans, this technique could be applied via laparotomy, laparoscopy, and percutaneous route. As the ultimate objective in gene therapy is the application to human diseases, this selective adenovirus-mediated gene delivery approach to specific liver segments may pave the way for future clinical applications in the treatment of focal liver disease, such as metastases.

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