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Intra-luminal gene therapy in the porcine artery using a recombinant adeno-associated virus 9

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Abstract

The ability to improve or restore blood flow and promote healing in ischemic tissue has many potential clinical applications. Augmentation by direct delivery of growth factors may further enhance results, but requires a method for sustained delivery. In this study, we have tested the ability of adeno-associated virus 9 (AAV9) delivered within the lumen of a porcine artery to transfect the vessel and produce a desired product. The marker chosen was green fluorescent protein (GFP) [1].

In 4 farm pigs the cranial tibial artery was surgically exposed. The vessel was temporarily clamped proximally, and divided distally. A cannula was placed intraluminally, and the arterial segment was injected with 1×10^{13} particles of AAV9.CB7.CI.GFP.WPRE.rBG. At 14 days the transfected cranial tibial artery as well as the liver, spleen and kidneys were harvested. ELISA and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) were used to analyze the artery for GFP production.

Significant GFP expression was seen in all transfected cranial tibial vessels, as determined by both GFP protein production (ELISA) and mRNA (RT-qPCR). No GFP was identified in liver, spleen or kidney, nor in the no-GFP control animal artery.

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Adeno-associated virus 9 is an appropriate vector for gene therapy experiments in the porcine artery model. This vector, and the intraluminal deliver method described results in robust gene expression at 2 weeks without evident systemic spill of the virus. The ability to limit delivery of the gene to an isolated segment of vessel is desirable for future research applications.

Keywords

adeno associated virus; gene therapy; vascular disease; porcine

Introduction

Ischemic tissues are dependent on healthy blood flow for healing. Surgical angiogenesis by implanting vessels or vascularized tissue into these areas of poor perfusion have shown to have beneficial effects on revascularization. Local delivery or production of growth factors may be useful to augment and accelerate revascularization and tissue healing. Gene therapy rather than direct delivery of proteins is a particularly attractive modality, as it may accomplish sustained and localized production of these growth factors. Adeno-associated virus (AAV) is proving to be of value in human gene therapy due to its safety, with no apparent pathogenicity, high transduction efficiency and sustained production of the transfected gene [1]. AAVs have, with 12 different serotypes [1] that differ primarily in transfection of different tissue targets. It has a single stranded DNA genome and is the smallest known virus [2–3]. Prior exposure to wild AAVs may occasionally result in the presence of neutralizing antibodies, which may limit or prevent effective gene therapy [2]. In rabbits, vascular endothelial transfection has demonstrated only a low transfection efficiency. Among the serotypes that were used were AAV1 and AAV5 [4–5]. In the cited studies, use of a higher viral load (1×10^{11} viral particles) resulted in better transfection, while delivery of 1×10^9 particles showed little or no transfection. We hypothesized that transfecting porcine artery with AAV9 in a high dose (1×10^{13} particles) into a short segment of artery would reliably transfect the porcine vessel. We chose AAV serotype 9 because of its reportedly rapid onset of gene expression, wider range of reliably transfected tissues, and high transfection potency [6]. The study aimed to demonstrate method efficacy prior to planned studies of gene therapy in the pig model. Green fluorescent protein (GFP) was used as the reporter gene as it does not occur naturally in porcine tissue and is easily detected.

Methods

AAV Vector

Recombinant adeno-associated virus 9 (AAV9) expressing green fluorescent protein (GFP) was constructed and produced by the Penn Vector Core at the University of Pennsylvania, using chicken beta actin (CB7) as a promoter, and woodchuck hepatitis post-transcriptional regulatory element (WPRES) and Chimeric Intron (CI) to increase gene expression. In the AAV cis plasmid, a rabbit beta-globin polyadenylation sequence is placed after WPRES to ensure transcription termination and mRNA stability. On mRNAs, the poly(A) tail protects the mRNA molecule from enzymatic degradation in the cytoplasm and aids in transcription

termination, export of the mRNA from the nucleus, and translation. The completed vector is therefore identified as AAV9.CB7.CI.GFP.WPRE.rBG with a titer of 5.534×10^{13} genome copies/ml (GC/ml), obtained using the droplet digital PCR (ddPCR)-method [7].

Animals

Four domestic pigs (50lbs) were used in this study (Manthey Hogfarm, Elk River, MN), following approval by the Institutional Animal Care and Use Committee.

Surgical procedure

Under general inhalational anaesthesia a 15cm anterolateral hind limb incision was made between the right knee and ankle joint. The anterior and lateral compartment muscles were reflected from the lateral margin of the tibia to expose the cranial tibial vessels. The cranial tibial artery and venae comitans were clamped proximally and divided 5 cm distal to this point. A 24 gauge I.V. catheter (Jelco, Dublin, Ohio) was introduced into the distal cranial tibial artery [fig 1]. It was used to inject 1×10^{13} particles of AAV suspended in 0.6 ml of PBS, 35mM NaCl and 0.001% Pluronic F68 into the arterial lumen over a period of 20–40 s. The proximal clamp was left in place for 30 minutes to allow transfection of endothelial cells without arterial inflow. The clamp and catheter were then removed and the distal infusion site ligated. The incision was closed in a layered fashion.

Postoperative Care

The animals received infection prophylaxis for 14 post-operative days, consisting of ceftiofur (Excede®, Zoetis, Parsippany, NJ), 5mg/kg and enrofloxacin every 5 days (Baytril®, Bayer Pharmaceuticals Leverkusen, Germany), 7.5mg/kg every other day by intramuscular injection.

The animals were euthanized 14 days post transfection with Fatal Plus® (Vortech pharmaceuticals, Ltd. Dearborn, MI), 100mg/kg intravenously. Samples from the treated cranial tibial artery, liver, spleen and kidneys were collected. Identical tissues were collected from an untreated farm pig as a no-GFP control.

Neutralizing antibody testing

Neutralizing antibody testing was performed by the Department of Molecular Medicine at Mayo Clinic. Blood (3ml) was drawn from each pig prior to surgery. Three nude mice/pig were injected intraperitoneally with 100µl of pig serum. The next day 1×10^{11} p AAV9-hCEA (human Carcinoembryonic antigen) was injected similarly. Two additional mice in total were used as negative and positive controls. The negative control mouse was injected intraperitoneally with PBS on day 1, followed by 1×10^{11} p AAV9-hCEA on day two. The positive control was injected with dog serum known to have neutralizing antibodies against AAV9 (Department of Molecular Medicine at Mayo Clinic, Rochester MN) followed by 1×10^{11} p AAV9-hCEA on day 2. 14 days after the mice were injected with pig serum, their blood was tested for hCEA. The hCEA serum levels of the 12 mice injected with pig serum were compared to the high hCEA values of the negative controls and low hCEA values in positive control mice. If the hCEA levels of the tested animals were 50% less than the negative control it can be assumed there are neutralizing antibodies present.

Gene transfer analysis

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used for GFP mRNA detection in the cranial tibial artery. We also similarly examined the liver, spleen and kidneys for evidence of systemic viral spread. RNA extraction was accomplished by crushing the tissues in liquid nitrogen with a hammer. RNA extraction was completed using an RNA extraction kit (BioChain Institute, Inc., Newark, CA). RNA was transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Primer sequences for GFP were forward 5'-CAAGATCCGCCACAACATCG-3' and reverse 5'-GACTGGGTGCTCAGGTAGTG-3'. RT-qPCR was performed using SYBR green (Qiagen, Hilden, Germany) and the CFX384 Real-Time System (BioRad, Hercules, CA). The cycling conditions used were 15 min at 95°C followed by cycles of 15s at 95°C and 30s at 60°C. Measurements were normalized to GAPDH, and gene expression levels were quantified using the 2^{-C_t} method, according to Lival et al [8].

An enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, MA) was used to quantify the GFP protein levels in the cranial tibial artery. Prior to use the collected tissue (100mg of tissue per pig) was disrupted using a mortar and pestle and adding extract buffer solution from the kit. The ELISA kit protocol was followed. 50 µl of sample and 50 µl of antibody cocktail was added to each well. The plate was incubated for 1 hour at room temperature and mixed on a plate shaker at 400rpm. Each well was washed with 3x 350 µl 1x Wash Buffer PT. 100 µl of TMB (Tetramethylbenzidine) substrate was added to each well and incubated for 10 min in the dark on the shaker. Stop solution (100 µl) was added to each well and put on a plate shaker for 1 min. The data were analyzed by reading the absorbance of the wells at 450nm using a plate reader (Tecan Group, Maennedorf, Switzerland). The absorbance of the tested samples was compared to the absorbance of the standard curve. The data was obtained by using the Magellan Data Analysis Software.

Results and discussion

Prior to surgery the 4 treatment pigs were tested for neutralizing antibodies against AAV9 as described above. Only serum from Pig 1 had levels of hCEA indicating pre-existing anti-AAV9 antibodies. (Fig.1).

ELISA of cranial tibial artery for GFP protein confirmed successful transfection in all pigs. Two of the treated cranial tibial arteries from pig 1 and 4 contained more than 2000 pg/ml of GFP, the highest detectable range of the ELISA kit, and despite the presence of neutralizing antibodies against AAV-9 in Pig 1. Pig 2 had GFP levels of approximately 500 pg/ml and pig 3 of approximately 1000 pg/ml. The no-GFP control artery contained no GFP (Fig. 2). Gene expression correlated well with GFP protein detection. We found GFP mRNA at levels many times higher than that for GAPDH (housekeeping gene) (Fig. 3). Again, GFP expression in pig 1 did not seem adversely effected by neutralizing antibodies against AAV-9. There was no GFP expression in any liver, spleen or kidney, indicating there was no or limited virus spread.

In this study we used adeno-associated virus 9 (AAV-9) as a vector for gene therapy in order to surgically transduce porcine peripheral artery with green fluorescent protein (GFP). We

used a dose of virus higher than other reports in blood vessel, based upon a published dose response curve in a similar porcine model [8]. We found the vector and route of administration together to permit effective transduction in porcine arteries, even in the presence of neutralizing antibody preoperatively. This finding in one animal is of uncertain significance, as others have found lower transfection rates when anti-AAV antibodies are present before viral delivery [2].

Conclusion

Adeno-associated virus 9 delivered by intraluminal injection is an appropriate method for gene therapy in porcine blood vessels. High levels of GFP protein and mRNA were seen in transfected arteries two weeks after gene delivery. Intraluminal delivery of the virus into an occluded vessel segment, with a 30 minute incubation period and subsequent distal vessel ligation resulted in a high level of gene expression, without evident systemic spill of the virus.

Abbreviation list

AAV	adeno associated virus
CI	Chimeric Intron
GFP	green fluorescent protein
hCEA	human Carcinoembryonic antigen
rBG	rabbit beta globin
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
TMB	Tetramethylbenzidine
WPRE	woodchuck hepatitis post-transcriptional regulatory element

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Highlights

- Adeno associated virus 9 (AAV9) is an appropriate vector for gene therapy in porcine peripheral artery.
- Presence of pre-existing antibodies to AAV9 does not still result in high gene production after 14 days.
- Using our surgical transfection method where we temporarily occlude the artery proximally with a microarterial clamp, infuse the AAV intraluminally, then ligate the pedicle distally. No evidence of viral spill by GFP production was seen in the liver, spleen and kidneys of 4 transfected pigs.

Fig 1. surgical procedure; ligation of distal artery and transfection of adeno associated virus (AAV) containing green fluorescent protein.

Fig 2. Neutralizing antibodies nanogram per milliliter (ng/ml). Human Carcinoembryonic antigen (hCEA) Negative control (mice injected with PBS) positive control (mice injected with dog serum known to have neutralizing antibodies against AAV9)

Fig 3.
ELISA data pictogram per ml lysate (pg/ml), 100 mg of tissue was used per pig

Fig 4. real time PCR data Relative expression units [REU]. Green fluorescent protein (GFP)